HYDROGEN PEROXIDE-INDUCED CELL DAMAGE:
THE ROLE OF FREE RADICALS AND IRON COMPLEXES

by

Sonja K. Jonas, BSc

Department of Biology and Biochemistry

Brunel University, Kingston Lane, Uxbridge UB8 3PH

submitted for the degree of Doctor of Philosophy

November 1988

ABSTRACT

This thesis presents data exploiting one of the important chemico-biological characteristics of the transition metal iron: its ability to exist in two oxidation states. Manipulation of the reactivity of iron by ligands has been reflected by the oxygen utilisation or the the amount of ferrozine-detectable Fe^{2+}.

Ligand-dependent cell associated iron has been demonstrated by prussian blue staining and ferrozine-detectable iron. Iron/8-hydroxyquinoline enters the cell by diffusion, whereas iron dextran is endocytosed as is evident from extensive vesicular staining.

The involvement of iron in the cytotoxicity of H_2O_2 has been examined (a) indirectly by introduction of reducing agents which restore the cytotoxicity of H_2O_2 at 4°C (an effect which is abolished by desferrioxamine) and (b) directly by extracellularly added iron, which enhances the cytotoxicity if the iron is internalised but protects the cell if the iron remains extracellular.

Cytotoxicity has been estimated by plating efficiency and by a modified tritiated thymidine incorporation assay permitting a 24 hour delay before exposure to the label.

Direct free radical interaction with a ligand itself is exemplified by the production of the nitroxide free radical in desferrioxamine. This causes extensive damage to yeast alcohol dehydrogenase but can be protected by ascorbate, methionine and iron/EDTA but not iron dextran.

The findings lend further support for the suggestion that free radical generation by the Fenton reaction enhances the potential cytotoxic effect of H_2O_2 provided the reactions occur at critical sites within the cell.

ACKNOWLEDGEMENTS

I would first like to express my gratitude to Professor R.L.Willson who gave me the opportunity to gain experience in several fields of free radical research and was supportive and encouraging throughout.

I am indebted to Professor P.A.Riley whose unfailing enthusiasm and guidance helped to focus an initially difficult task towards an interesting and encouraging project.

I wish to express my thanks to Dr.C.Bell whose contribution and discussion helped clarify some of the complicated chemistry involved.

I am most grateful to the staff of the Department of Chemical Pathology of University College/Middlesex School of Medicine as well as the Free Radical Group of Brunel who were consistently helpful and stimulating and enabled me to undertake this work.

I thank all those friends whose lasting interest was encouraging.

Finally, I gratefully aknowledge the financial support of Fisons Pharmaceuticals Plc.

CONTENTS Page no.

Abstract 2
Acknowledgements 4
Contents 5
List of tables 11
List of figures 13
List of Plates 16
List of Abbreviations 17
CHAPTER I: INTRODUCTION AND AIMS OF THESIS
1.1 Iron in living organisms 20
1.2 Iron Absorption 20
1.3 Iron Proteins 20
1.4 Iron mobilisation 22
1.5 Non-protein-bound 'iron-tansit pool' 23
1.6 Iron deficiency and treatment 25
1.6.1 Iron Dextran 26
1.7 Iron overload and treatment 28
1.8 Basic physical,chemical and biochemical properties of iron 30
1.8.1 Autoxidation of Fe(II) 31
1.8.2 Hydrolysis of Fe(III) 33
1.9 Iron and free radicals 34
1.9.1 Fenton Reaction 34
1.9.2 Tissue damage 35
1.9.3 Alternatives to the hydroxyl radical 38
1.9.4 Reactions of iron with thiols 39
1.10 Hydrogen peroxide 40
1.10.1 Toxicity 40
1.10.2 Cytotoxicity 42
CHAPTER II: CHEMICAL STUDIES ON THE REACTIONS OF IRON COMPLEXES AND HYDROGEN PEROXIDE
2.1 INTROCUCTION 45
2.1.1 Effects of ligands on the chemistry of iron 45
2.1.2 EDTA (ethylenediaminetetraacetic acid) 47

2.1.3 DTPA (diethylenetriaminepentaacetic acid) 47
2.1.4 Heterocyclic nitrogen-type ligands 49
2.1.5 Ferrioxamines 49
2.1.6 Iron dextrans 51
2.1.6.1 Manufacture of iron dextrans 52
2.1.7 Redox state of iron 53
2.2 MATERIALS AND METHODS 54
2.2.1 Solutions of iron salts 54
2.2.2 Solutions of ligands used for iron 54
2.2.3 Preparation of iron complexes 54
2.2.4 Iron Dextrans 55
2.2.5 Buffer solutions 55
2.2.5.1 Phosphate buffered saline 55
2.2.5.2 Imidazole buffer 55
2.2.6 Hydrogen peroxide solutions 56
2.2.6.1 Hydrogen Peroxide Assay 56
2.3 RESULTS 59
2.3.1 Partition coefficients 59
2.3.2 Effects of ligands on the redox state of iron 61
2.3.2.1 Measurement of O_2 consumption by iron complexes 62
2.3.2.2 Ferrozine-Fe(II) complex 65
2.3.2.3 Comparison of the oxidation rates from both assays 70
2.3.2.4 Comparison of uncomplexed and complexed Fe(II) by phenanthroline or ferrozine 72
2.3.2.5 Autoxidation of Fe/dextrans as measured by ferrozine 76
2.3.2.6 Reduction of Fe/dextran by ascorbate 76
2.3.2.7 Effect of ascorbate on the rate of autoxidation of Fe/EDTA 81
2.3.2.8 Effect of ascorbate and H_2O_2 on the rate of O_2 consumption 81
2.3.2.9 H_2O_2 concentration 89

2.3.2.10 Half-life of H_2O_2 in the presence of iron complexes 94
2.4 DISCUSSION 97

CHAPTER III: LOCALISATION OF IRON IN CELLS AND ITS EFFECT ON SURVIVAL

3.1 INTRODUCTION 102
3.2 MATERIALS AND METHODS 102
3.2.1 General cleaning procedure 102
3.2.2 Sterilising 103
3.2.3 Culture medium 103
3.2.4 Cell line 104
3.2.5 Freezing 104
3.2.6 Autoradiography 105
3.2.6.1 Coating 105
3.2.6.2 Developing 106
3.2.6.3 Staining 106
3.2.7 Trypsin 106
3.2.8 Versene 107
3.2.9 Details of subculture 107
3.2.10 Doubling time 108
3.2.11 Plating efficiency: colony formation 108
2.1.12 Thymidine Incorporation Assay 108
3.2.12.1 Backround 110
3.2.12.2 Detection of label 110
3.2.12.3 Labelling of cells 110
3.2.13 Statistical tests 113
3.2.13.1 Student's T-test 113
3.2.13.2 Linear regression analysis 114
3.3 RESULTS 115
3.3.1 Quantitative estimation of cell-associated iron 115
3.3.2 Iron staining (Prussian Blue) 119
3.3.3 Staining for dextran 120
3.3.4 Effects of iron complexes on cell survival 126
3.3.4.1 ^{3}H-TdR incorporation 126
3.3.4.2 Plating efficiency 130

3.3.4.2.1 Effects of different Fe/dextrans 130
3.4 DISCUSSION 133
CHAPTER IV: STUDIES WITH HYDROGEN PEROXIDE USING MAMMALIAN CELLS
4.1 INTRODUCTION 135
4.2 MATERIALS AND METHODS 140
4.2.1 Reducing agents 140
4.2.1.1 Ascorbic acid 140
4.2.1.2 Lipoic acid 140
4.2.2 Zinc salts 140
4.2.3 Cytotoxicity assays 141
4.2.3.1 Exposure of cells to H_2O_2 at different tempertures 141
4.2.3.2 Modification of H_2O_2 cytotoxicity by various agents 142
4.3 RESULTS 143
4.3.1 Half-life of H_2O_2 in the presence of cells 143
4.3.1.1 Addition of glucose 149
4.3.1.2 Effects of 3-amino-1,2,4-triazole (3-ATZ) 152
4.3.1.3 Effects of penicillamine (PCA) 155
4.3.1.4 Effect of pH 158
4.3.1.5 Effect of boiled cells 158
4.3.1.6 Effect of temperature 161
4.3.2 Cytotoxicty assays 161
4.3.2.1 Plating efficiency (P.E.) 164
4.3.2.2 ^{3}H-TdR incorporation 164
4.3.2.2.1 Passage number 164
4.3.2.2.2 Survival Index (S.I.) 166
4.3.2.3 Comparison of P.E. with S.I. 168
4.3.2.4 Effect of exposure to H_2O_2 on cell viability studied by (S.I.) 171
4.3.2.5 Effects of addition of glucose 174
4.3.2.6 Effect of pre-incubation with 3-ATZ 176
4.3.2.7 Effect of PCA 179

4.3.2.8 Effect of pH 181
4.3.2.9 Effects of temperature on the S.I. 185
4.3.2.9.1 Effects of ascorbic acid 185
4.3.2.9.2 Effects of lipoic acid 189
4.3.2.9.3 Protection by desferral 189
4.3.2.10 Effects of zinc 192
4.3.3 Modification of H_2O_2-induced cytotoxicity 197
4.3.3.1 Effects of Fe/EDTA (P.E.) 197
4.3.3.2 Effects of Fe/dex (P.E.) 203
4.3.3.3 Effects of iron complexes on H_2O_2 cytotoxicity (^3H-TdR incorporation) 203
4.3.3.4 Simultaneous exposure to iron complexes and H_2O_2 205
4.3.3.5 Pre-incubation with iron complexes 205
4.3.3.5.1 Effect of iron dextran 209
4.3.3.5.2 Effect of Fe/ATP 211
4.3.3.5.3 Effect of Fe/8-HQ 211
4.3.3.6 Effect of zinc aspartate 211
4.3.3.7 Effect of pre-heating iron dextran with ascorbate 216
4.4 DISCUSSION 220
4.4.1 Degradation of H_2O_2 by cells 220
4.4.2 Mechanism of H_2O_2-induced cytotoxicity 224
4.4.3 Effects of iron complexes 233
CHAPTER V: DIRECT INTERACTION OF FREE RADICALS WITH A LIGAND AND THE EFFECT ON YEAST ALCOHOL DEHYDROGENASE (YADH)
5.1 INTRODUCTION 239
5.1.1 The structure and function of YADH 240
5.1.2 Radiation induced free radical generation 241
5.1.2.1 Formation of primary products from water radiolysis 242
5.1.2.2 Reactions of primary products 243
5.1.2.3 Reactions of secondary radicals 246
5.2 MATERIAL AND METHODS 248
5.2.1 Gamma irradiation source 248

5.2.2 YADH assay 248
5.2.3 Irradiation of all solutes present 248
5.3 RESULTS 253
5.3.1 Inactivation of YADH by radiation 253
5.3.1.1 Reactions with superoxide ($O_2^{\cdot -}$) and desferrioxamine (DFO) 253
5.3.1.2 Effect of thymine peroxy radical on the activity of YADH 260
5.4 DISCUSSION 266
CHAPTER VI: GENERAL DISCUSSION AND CONCLUSIONS 273
APPENDIX I: Student's T-test 280
APPENDIX II: Linear regression analysis 281
APPENDIX III: Calculation of degradation rate (k) of H_2O_2 284
APPENDIX IV: Calculation of total dose (D) in μM mins 285
APPENDIX V: Steady-state radiolysis and dosimetry 286
REFERENCES 289

LIST OF TABLES Page no.

2.1 Partition coefficients of iron complexes 60
2.2 Rates of autoxidation by iron complexes 73
2.3 Fe^{2+} detection by phenanthroline or ferrozine 74
2.4 Fe^{2+} detection by phenanthroline or ferrozine in Fe(II)/ADP (1:20 complex) 75
2.5 Detection of iron as Fe(II) in ascorbate-reduced Fe/dex by phenanthroline or ferrozine 77
2.6 Variation of Fe(II) content in iron dextran batches exposed to air for different times 78
2.7 H_2O_2 concentration remaining upon addition of Fe/EDTA and/or ascorbate 91a
2.8 Examples of stability constants of some iron complexes 99
3.1 Iron concentration in cells treated with different iron complexes 117
3.2 Ferrozine-detectable iron from cells treated with iron dextran 118
3.3 Effect of ligands in the presence and absence of $FeSO_4$ on ^3H-TdR incorporation 128
3.4 Survival of cells in the presence of iron complexes 131
4.1 Half-life of H_2O_2 as a function of cell density 147
4.2 Recovery of cells 24 hours after treatment with H_2O_2 151
4.3 Effect of D-glucose on recovery of cells 24 hours after exposure to H_2O_2 154
4.4 Effect of pre-treatment with 3-ATZ on recovery of cells 24 hours after exposure to H_2O_2 157
4.5 Variation of thymidine incorporation with passage number of CNCM I-221 cells 167
4.6 Effect of D-glucose on the cells exposed to H_2O_2 177
4.7 Restoration of the cytotoxicity of H_2O_2 at 4°C by pre-incubation with L-ascorbate 187

4.8 Restoration of the cytotoxicity of H_2O_2 at 4°C by dehydroascorbate 188
4.9 Effect of pre-incubation with lipoic acid on the absence of cytotoxicity at 4°C by H_2O_2 190
4.10 Effect of desferrioxamine (DFO) on the restoration of H_2O_2-induced cytotoxicity at 4°C by ascorbate 194
4.11 Effect of ZnHis on the % S.I. in the presence of H_2O_2 196
4.12 Effect of ZnAsp on the % S.I. in the presence of H_2O_2 199
4.13 Increased cytotoxicity of H_2O_2 in the presence of Fe/dex 204
4.14 Effects of iron complexes on the cytotoxicity of H_2O_2 (100μM Fe, 500μM ligand) 207
4.15 Effect of pre-incubation with Fe/dex on H_2O_2-induced cytotoxicity 210
4.16 Effect of batches of iron dextran on the cytotoxicity of H_2O_2 211
4.17 Effect of pre-incubation with Fe/ATP on the H_2O_2-induced cytotoxicity 214
4.18 Effect of ZnAsp on the Fe/dex enhanced H_2O_2 cytotoxicity 217
4.19 Restoration of the toxicity of H_2O_2 by pre-incubation with pre-heated ascorbate and Fe/dex 219
4.20 Types of DNA damage caused by oxidative stress 227
5.1 Protection of YADH from the nitroxide radical by ascorbate 261
5.2 YADH inactivation by DFO at the time of irradiation in the presence or absence of formate 262
5.3 Protection of YADH from the thymine peroxy radical by Fe(III)/EDTA 264
5.4 The effect of DFO on YADH inactivation by the thymine peroxy radical; protection by Fe(III)/EDTA 265

LIST OF FIGURES   Page no.

2.1 Structure of EDTA 48

2.2 Structure of DTPA 48

2.3 Structures of some heterocyclic N-compounds which bind iron 50

2.4 Calibration curve for KI oxidation by H_2O_2 58

2.5a,b Oxygen utilisation rate by $FeSO_4$ in the presence of ligands 63/64

2.6 Structure of ferrozine 66

2.7 Calibration curve for ferrozine with $FeSO_4$ in water 68

2.8 Saturation curve for ferrozine with 100μM $FeSO_4$ 69

2.9 Rate of oxidation of Fe^{2+} bound to different ligands 71

2.10 Measurement of Fe(II)/ferrozine from Fe/dextran exposed to air for different times 79

2.11 Rate of formation of Fe/ferrozine complex from Fe/dextran in the presence of ascorbate 80

2.12 Effect or pre-heating Fe/dextran with ascorbate on the formation of the Fe/ferrozine complex 82

2.13 Rate of O_2 consumption in the presence of $FeCl_3$/EDTA +/- ascorbate 83

2.14 Rate of of O_2 utilisation by $FeCl_3$ complexes in the presence of H_2O_2 and ascorbate 85

2.15 Rate of O_2 utilisation by $FeSO_4$ complexes in the presence of H_2O_2 and ascorbate 86

2.16 Rate of O_2 consumption with increasing concentration of Fe/EDTA 87

2.17 Rate of O_2 utilsation in the presence of H_2O_2, ascorbate and Fe/dex 88

2.18 pH profile of Fe/dex in the presence of H_2O_2 and ascorbate 90

2.19 Effect of changing the concentration of H_2O_2 or ascorbate on the rate of O_2 utilisation 91

2.20 Effect of ascorbate concentration on the O_2 utilisation 92

2.21 Effect of pH on the half-life of O_2 in the ascorbate/H_2O_2/$FeCl_3$/EDTA system 93

2.22 Rate of removal of H_2O_2 by Fe(II)/EDTA or Fe(II)/ATP 95

3.1 Effect of 8-HQ on cell viability in the presence of Fe salts 129

4.1 Metabolic Integration of catabolism of H_2O_2 by glutathione peroxidase (GPx) 136

4.2 Decay of H_2O_2 in the presence of cells 145

4.3 Rate of H_2O_2 removal with varying cell number 148

4.4 Total dose of H_2O_2 as a function of cell number 150

4.5 Effect of glucose on the rate of H_2O_2 decay by cells 153

4.6 Effect of pre-incubation of cells with 3-ATZ on H_2O_2 degradation 156

4.7 Effect of PCA on the degradation of H_2O_2 by cells 159

4.8 Effect of pH on the rate of H_2O_2 degradation by cells 160

4.9 Effect of boiled cells on the rate of H_2O_2 removal 162

4.10 Rate of H_2O_2 removal by cells at 37°C and 4°C 163

4.11 Survival of CNCM-221 cells exposed to H_2O_2 165

4.12 Survival index (S.I.) as a function of H_2O_2 concentration 169

4.13 Correlation of ^{3}H-TdR incorporation with plating efficiency 170

4.14 % S.I. as a funtion of cell no. at various concentrations of H_2O_2 172

4.15 Regression plot % S.I. as a function of total dose of H_2O_2 173

4.16 Regression line for S.I. with increasing dose of H_2O_2 175
4.17 Effect of 3-ATZ on the S.I. of cells 178
4.18 Effect of PCA on the S.I. of cells 180
4.19 Effect of altering pH on the S.I. of cells 182
4.20 Regression line for S.I. with at 37°C and 4°C 184
4.21 Effect of temperature on the S.I. of 221-CNCM cells in the presence of H_2O_2 185
4.22 Dose-dependent protection by DFO 195
4.23 Effect of ZnHis on the S.I. of cells 198
4.24 Effect of ZnAsp on the S.I. of cells 200
4.25 Effect of Fe(II)/EDTA and H_2O_2 on cell survival 202
4.26 Effect of Fe/dex and H_2O_2 on the S.I. of cells 213
4.27 Effect of Fe/8-HQ on the S.I. of cells 215
4.28 Relationship between the ratio of CPM to $O.D._{280}$ and cell density 225
4.29 Oxidation of ascorbic acid 229
4.30 Schematic diagram of the intracellular events leading to the restoration of H_2O_2-induced cytotoxicity at 4°C. 231

5.1 Plan of the Brunel ^{60}Co gamma radiation facility 249
5.2 Protection of YADH by formate 255
5.3 Inactivation of YADH by DFO in the presence of formate 256
5.4 Effect of dose of DFO on the inactivation of YADH 258
5.5 Effect of amino acids on the inactivation by the DFNO· radical 259

LIST OF PLATES

Plates I to IV A show prussian blue staining of CNCM I-221 cells

PLATE I: Fe/8-hydroxyquinoline
PLATE II: Fe/dextran
PLATE III: "
PLATE IV A: "
PLATE IV B: FITC (fluorescent) dextran

LIST OF ABBREVIATIONS

ADP	adenosine 5'diphosphate
ASC	ascorbic acid
3-ATZ	3-amino-1,2,4-triazole
ATP	adenosine 5'triphosphate
DHA	dehydroascorbic acid
DFO	desferroxamine
dex	dextran
DTPA	diethylenetriaminepentaacetic acid
DSB	double strand breaks
E.F.	enhancement factor
EDTA	ethylenediamine tetracetic acid
FZ	ferrozine ([3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonic acid)1,2,4-triazine
GPx	glutathione peroxidase
8-HQ	8-hydroxyquinoline
Im buffer	Imidazole buffer
LPA	lipoic acid
NAD	nicotinamide adenine dinucleotide
o,phen	o,phenanthroline
P/C	partition coefficient
PCA	penicillamine (β-mercaptoethanol)
PBSi	phosphate buffered saline for incubation
P.E.	plating efficiency
SC MEM or MEM	serum containing growth medium
S.I.	survival index

SSB	single strand breaks
^{3}H-TdR	[methyl-^{3}H]-Thymidine
TCA	trichloroacetic acid
YADH	yeast alcohol dehydrogenase
ZnAsp	zinc aspartate
ZnHis	zinc histidine

CHAPTER I: INTRODUCTION

1.1 Iron in the living organism

Both the abundance and the range of biochemical reactions of iron in the living organism lead to its indispensable role in life. To name only a few examples: it is involved in the transport of oxygen by haemoglobin (Hb) and haemerythrin; in electron transfer reactions; in the synthesis of DNA (as an essential component of ribonucleotide reductase); in the catalysis of oxidation by oxygen and hydrogen peroxide etc. Its essentiality has led to the conclusion by Neilands (1972) that "life, in any form, without iron, is in all likelihood impossible".

Total body iron, estimated on the basis of individual fractions varies between 25-75mg/kg body weight. An 80kg male has about 4g of iron and the iron content for a 65kg female is approximately 2.5g (Bothwell & Finch, 1962). Most of the quantitatively estimated iron is found in Hb, ferritin-haemosiderin and myoglobin, of which Hb constitutes the largest fraction of body iron. It accounts for between 60-80% of essential iron (Cook, 1970). Additionally, many other cellular processes are iron dependent, generally because within the physiological pH range appropriately coordinated iron atoms can exist in either the ferric or ferrous form and are therefore well suited for use in biochemical reactions which involve electron transport (Wrigglesworth

& Baum, 1980).

1.2 Iron Absorption

Iron transport across intestinal brush-boarder membranes, the first step in iron absorption, has been extensively studied. It is thought that specific cellular membranes allow passage of iron across them and lipids have been implicated as a possible carrier in brush-boarder membrane vesicles (Simpson & Peters,1987).

Most dietry iron is in the ferric state, but only ferrous iron is absorbed from the gastrointestinal tract. Some of the ferric iron is reduced to ferrous in the presence of acid in the stomach, and absorption takes place mainly from the duodenum and jejenum. In the mucosal cells of the small intestine ferrous iron is converted to ferric for binding to transferrin. At any time only about 30% of the iron binding sites available on plasma transferrin are occupied (Sullivan,1985).

1.3 Iron proteins

Although iron is abundant within the earth's crust, it is not always present in a form which can be easily assimilated by living cells (Finch & Huebers,1982). Large amounts of stored iron are required for the synthesis of Hb and other proteins. However, it is difficult to accumulate large amounts of iron *in vivo*, because hydrated ferric ions are converted to insoluble ferric oxide at physiological pH (solubility product,Ks, for iron is approximately 38) (Biedermann & Schindler,1957).

Such precipitates are potentially toxic to cells (Harrison & Hoy,1973). For this reason virtually all organisms have evolved specific iron-binding molecules (Aisen & Listowski,1980).

Transferrin has been regarded as one if not the most important transport plasma glycoprotein in vertebrates and it also has a high affinity for iron. It binds ferric iron firmly between imidazole and tyrosine residues (Aasa et al.,1963). Transferrin normally circulates in a partially unsaturated form so that it can mop up any free iron present in body fluids. The total amount of transported iron is only about 3-4 mgs, ie about 1% of total body iron (Bernat,1983). Studies have shown that cells regulate their iron requirements by the number of transferrin receptors on the cell (Iacopetta et al.,1982).

In vitro , the release of iron from transferrin is most readily achieved by lowering the pH to values between 5-5.5 in the presence of appropriate Fe(III) chelators and it has been reviewed that the physiological consequences of the inclusion of transferrin within acidic intracellular vesicles results de facto in the release of iron within the cell (Crichton & Charloteaux-Waters,1987; Jin & Crichton,1987). The mechanism of transfer of iron from transferrin to ferritin is still controversial, but it is generally agreed that the process involves a change in oxidation state of the iron.

As apoferritin more readily assimilates Fe(II) *in vivo* than Fe(III), the iron released from transferrin within the cells is Fe(III) and must therefore be reduced prior to incorporation into the storage protein (Hoy & Harrison,1976; Treffy & Harrison,1979).

Iron is stored in the body as ferritin and haemosiderin, which together represent the second largest fraction of iron after Hb (Awai & Brown,1963). Storage iron in these proteins varies in the adult male between 800mgs and 1.5g, but stores in women are much lower (Bernat,1983). Ferritin is found in the cytoplasm in a soluble form and in serum, but haemosiderin is present as insoluble granules within secondary lysosomes. The ratio between the two proteins differs according to the total amount of iron stored within the cell. At high iron concentrations most of the iron is found in haemosiderin (Drysdale *et al*.,1975).

1.4 Iron mobilisation

The mechanism of intracellular iron mobilisation is uncertain. In normal human subjects some 40mgs tissue iron is mobilised per day (Crichton,1984). It has previously been suggested that an inverse 'transferrin cycle' is operating to excrete iron (Dautry-Varsat *et al*.,1983; Klausner *et al*.,1983), but recently experiments have been carried out suggesting that the pathway involves excretion of ferritin (with iron) into the blood and transfer to apotransferrin (Jin & Crichton,1987). The

redox property of iron is also presumed to be of significance in the mobilisation process. Increasing evidence is pointing to the possibility that a reductive step is involved (Rowely & Sweeny,1984; Funk *et al*.,1985) and *in vitro* studies show that iron can be released from ferritin by reducing agents such as ascorbate, GSH, cysteine (Harrison,1964) and reduced flavins, which are considered to be the most likely reducing agent *in vivo* (Munro & Linder,1978). Further support for the involvement of reduced iron is provided by the finding that under certain circumstances iron can be released from iron-binding proteins and then facilitate the formation of hydroxyl radicals. This is shown by evidence for the stimulation of peroxidation of liposomes by ferritin (O'Connell *et al*.,1985) and peroxidation of liver and microsomal fractions (Koster & Slee,1986). Increasing agreement is emerging that iron is mobilised by xanthine oxidase (Biemond *et al*.,1984; Thomas *et al*.,1985; Bolam & Ulvik,1987) and moreover that it is superoxide which is responsible.

1.5 Non-protein-bound 'Iron-transit pool'

The intracellular iron pool is the focal point of intracellular iron metabolism. Its interaction with extracellular transferrin and similar transit pools in other tissues allows an equilibrium to be established not only between the various metabolic processes in the cell but also between different organs in the body (Carvill *et*

al.,1975). This non-haem, non-ferritin iron-transit pool is easily chelatable by desferrioxamine (DFO). The nature of the iron transit pool is unknown, except that it appears to be a low molecular weight, loosely bound complex of iron, or perhaps several such iron complexes. Candidates that have been suggested to act as transitional ligands include: ascorbate, ATP, citrate, glycine and lactose (Jacobs,1977). From experiments with cultured Chang cells which were incubated with [^{59}Fe]-transferrin about 70% of the iron was found in the cytosol, but about 35% was dialisable non-haem, non-ferritin bound iron (White et al.,1976). Direct estimation of the amount of iron in the cytosol by atomic absorption spectroscopy showed about 20fg/cell. After incubation of the cells for 7 days in a medium containing 170μM iron, the iron content of the cells was 100fg/cell. In 1969 Wills demonstrated the role of this potentially reactive iron in lipid peroxidation and it is probably of major importance in the toxicity found in overload conditions (Wills,1969).

The remainder of the cellular iron consists of non-haem metabolising active compounds with enzymatic function. In mitochondria these account for far more iron than do the cytochromes. A large proportion of these are designated as metalloflavoproteins and include reduced NAD, succinate and α-glycerolphosphate dehydrogenase (Bernat,1983).

1.6 Iron deficiency and treatment

Iron deficiency is the most prevalent world wide deficiency. In some countries it is almost universal and in different populations its incidence varies from 20-95% (WHO Technical Report,1959). In certain parts of Africa more than 50% of the population was found to be anaemic and between 10-40% of the maternal mortality during pregnancy and delivery in regions of India is attributable to iron deficient anaemia (Bernat,1983).

The mildest form of iron deficiency, depletion of iron stores, is characterized by the absence of storage iron. No other abnormalities are identifiable. The second stage, latent iron deficiency, is characterized by reduced serum iron concentration, elevated transferrin concentration, and a resultant drop in transferrin iron saturation to less than 15% (Bainton & Finch,1964). Concomitantly, haemoglobin synthesis is impaired by lack of iron, causing erythrocyte protoporphyrin concentration to rise to more than 70μg/dl (Langer et al.,1972). However, in this stage of iron deficiency, circulating Hb concentrations remain within the normal range (Garby et al.,1969). Overt iron deficiency, the third stage, is triggered when the residual Hb synthesis contributes to a measurable decrease in the concentration of circulating Hb or in the volume of packed erythrocytes.

Oral iron therapy is based on extensive studies

undertaken by Brise & Hallberg (1962) which compared the absorption from a variety of ferrous and ferric compounds with absorption from a standard preparation of ferrous sulphate using a double isotope technique. The majority of patients responded to simple oral iron medication. In general they found that ferrous sulphate ranked among the best iron compounds from the point of view of absorption. Although serious side effects with oral iron treatment are rarely encountered, there are sufficient indications, such as malabsorption or iron losses, to have resulted in the design of parenteral saccharated iron oxides. The first attempts at parenteral iron medication date back to the end of the nineteenth century, but the general and local side effects of the preparations rendered them unacceptable and it was not until 1947 when Nissim introduced a saccharated oxide of iron that a useful and safe preparation for parenteral use was found (Nissim,1947). Later iron dextran and iron sorbitol citrate were introduced. However, even with these preparations patients do not always escape side-effects. Reported are symptoms such as headaches and flushing (Fielding,1961; Newcombe,1967), fever, athralgia and lymhadenopathy (McCardy,1965).

1.6.1 Iron dextran (Fe/dex)

A few side-effects which are observed and which appear to be batch related still remain unaccounted for. These include anaphylactic responses (Hamstra *et*

al.,1980), exacerbation of rheumatoid arthritis (Reddy & Lewis,1969) and delayed athralgia and myalgia (Mittal *et al*.,1969). Some properties of Fe/dex were investigated in this thesis. Generally, however, its toxicity is comparatively low and thus it remains the safest iron preparation available for parenteral administration (Freed,1982).

Since Fe/dex has a high molecular weight, very little if any, of the complex is likely to enter the capillaries directly, and evidence indicates that it is absorbed via the lymphatics (Beresford *et al*.,1957). In response to tissue reactive absorption, diffusion is complete after 72 hours (Goldberg,1958). In experimental animals after Total Dose Infusion (TDI) of Fe/dex there was no evidence of saturation of iron binding capacity even when plasma iron values were greater than 1000µg/100ml (Cox *et al*.,1968). It has been suggested that the iron in the dextran does not easily dissociate from the dextran in the serum and thus the iron binding capacity remains unsaturated (Cox *et al*.,1965) with consequent low toxicity. This is in contrast to iron-sorbitol-citrate which is assumed to saturate or even exceed iron binding capacity with consequent high toxicity (Scott,1962). In the reticuloendothelial system iron is rapidly separated from the dextran, bound to transferrin or stored in ferritin or haemosiderin. Kanakakorn *et al*. (1973) found that about 40% of a dose

of intraveneously injected iron into the circulation was bound to transferrin 11 hours later.

1.7 Iron overload and treatment

In iron overload the amounts of both ferritin and haemosiderin become the predominant forms of iron (Selden *et al*.,1980). The excess iron is thought to catalyze peroxidation of the lysosomal membranes,thus allowing the lysosomal enzymes to leak into the cell causing further damage (Pierre *et al*.,1987). In serious cases where organs, containing grossly excessive amounts of storage iron, show pathological evidence of damage, usually fibrosis, the condition is referred to as haemachromatosis.

Iron poisoning has been frequently treated with desferrioxamine (DFO) and in chronic cases may have to be used for several years. It is regarded as a relatively safe drug for treating iron overload in patients receiving regular blood transfusions for chronic refractory anaemia. Children with β-thalassaemia major in Britain were first given DFO in 1962 and it appears to have been successful in prolonging their life-span (Modell *et al*.,1982). Both DFO and its complex (ferrioxamine) are rapidly excreted, mainly in urine but also in bile. However, large doses of DFO are required and it can not be given by mouth. The application of this metal-chelating drug is also fraught with difficulties. Side effects, affecting the sensory neurons, have already

been reported, many of which are severe (Blake,1985).

In a recent study by Barankiewicz (1987) DFO has been shown to inhibit ribonuclease reductase (an iron requiring enzyme) activity and as a consequence depletion of deoxyribonucleotides for DNA synthesis. Detailed studies on the effect of DFO on cell cycle showed that DFO acts as a reversible S phase inhibitor (Ledermann,1984). This effect has also been noted for a number of other hydroxamic acid derivatives incuding salicyl-, octano-, decano-, dodecano hydroxamic acid and rhodotorulic acid. These effects have been turned to an advantage in the consideration of this antiproliferative activity against leukemic cells (Ganeshagura *et al*.,1980).

As a tool in mechanistic free radical biochemistry, the drug may not only act as an iron chelator but may also scavenge free radicals directly. In particular it reacts rapidly with $HO\cdot$ ($K= 10^{10}\ M^{-1}s^{-1}$; Hoe *et al*.,1982; Willson,1982) as well as $O_2\cdot^-$ (Sinaceur,1983,1984). Some of these effects are described in this thesis.

The high expense and inconvenience of subcutaneous administration of DFO has led to the search of alternative iron chelators and recently Kontoghiorghes (1987) has introduced new compounds, the 2-hydroxypyridine N-oxide derivatives, which resemble an aromatic hydroxamate and which have proved successful in

causing ^{59}Fe excretion comparable to that of intraperitoneally administered DFO.

Other chelating agents have also been used in the past, such as EDTA and DTPA, given parenterally (Smith *et al.*,1969), but these are not specific to iron. Both are studied in this thesis.

1.8 Basic physical, chemical and biochemical properties of iron

Iron, in the form of various combined ores, is one of the most common elements, constituting 5% of the earth's crust. It is the fourth most abundant element, only O_2, silicon and aluminium are more common. Pure metallic iron is rare in nature; it is bluish white and strongly magnetic. It is unstable, being di-,tri-, or occasionally hexavalent. Its atomic number is 26 with an atomic weight of 52-61. Thus the nucleus contains 26 protons and 26-35 neutrons. The four stable iron isotopes have an atomic weight of 54,56,57 and 59 giving an atomic weight of the naturally occurring iron of 55.847. Around the atomic atomic nucleus of the iron 2+8+14+2=26 electrons circulate in 4 "shells" (Nicholls,1973).

Iron derivatives may be divalent ferrous compounds eg, $FeSO_4$, trivalent ferric compounds eg, $Fe_2(SO_4)_3$ or complex iron compounds eg, $K_4[Fe(CN)_6]$, in which iron is part of a complex anion (Bernat,1983).

In solution iron exists as Fe(II) or Fe(III) or as inorganic or organic ferrous or ferric complexes. It also

can be found in small quantities as a stable colloid or hydrosol, most commonly constituting small dispersed particles of ferric oxyhydroxide. Its composition is approximated by the formula $Fe(OH)_3$. This hydrolysis results in the formation of polynuclear hydroxide complexes which are biologically unavailable. Further factors which influence the redox equilibrium of iron in solution include oxygen tension, ionic composition and the presence of ligands which can form complexes with iron in one or the other principal oxidation states (Aisen,1977). The latter phenomenon is investigated in this thesis.

Because of the hydrolytic tendencies of ferric iron, the equilibrium concentration of Fe(III) in a physiological medium is usually too low to allow involvement of (hexaaquo) Fe(III) in biochemical reactions, including those of iron transport, at appreciable rates. Redox reactions, releasing iron as the relatively water-soluble Fe(II) may therefore be important in overcoming this kinetic barrier. Nature has been able to devise iron proteins with reduction potentials ranging from +350mV (the high potential iron-sulphur proteins or in the range of the strongest biological oxidants) to -500mV in other iron-sulphur proteins, making the reduced form a better reductant than hydrogen (Aisen,1977).

1.8.1 Autoxidation of Fe(II)

In a physiological milieu the simple equilibrium of 10^6 for:

$$4Fe^{2+}(aq) + O_2 + 4H^+ \rightleftharpoons 4Fe^{3+}(aq) + 2H_2O \qquad (1)$$

cannot apply. As the pH rises above 2, hydrolytic reactions predominate and the $Fe^{3+}(aq)$ species becomes virtually nonexistent. Goto etal.,(1970) have described the rate by:

$$-\frac{d[Fe^{2+}]}{dt} = K_0\ ([Fe^{2+}]\ [O_2]\ [OH^-]^2)$$

where K_0 is the overall equilibrium constant of the reaction. The equation has been subsequently attributed to a complicated and poorly understood sequence of events including the following reactions:

$$Fe^{2+} + OH^- \rightleftharpoons (FeOH)^+ \qquad (2)$$

$$O_2 + OH^- \rightleftharpoons (O_2OH)^- \qquad (3)$$

$$(FeOH)^+ + (O_2OH)^- \rightleftharpoons Fe(OH)_2^+ + O_2\cdot^- \qquad (4)$$

(Aisen,1977)

The existence of the hydroxylated oxygen molecule, O_2OH^-, as well as the iron(II) monohydroxide complex is assumed

to explain the quadratic dependence of the reaction rate on OH^- concentration.

1.8.2 Hydrolysis of Fe(III)

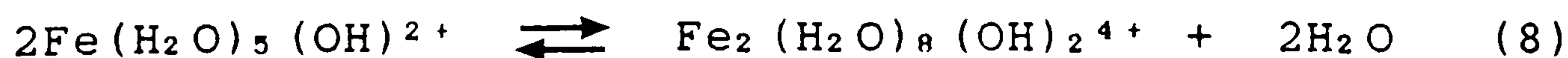
The hydrolysis of Fe^{3+} can be described by:

$$Fe(III) + 3OH^- \longrightarrow Fe(OH)_3\downarrow \quad (5)$$

for which the solubility product (log Ksp) is about 38. From this it can be calculated that the equilibrium concentration of Fe(III) at the pH of blood of 7.4 cannot exceed 10^{-18}M. Some 5 x 10^{-4} mol of iron are reported to turn over each day by the normal human subject and thus the need for a carrier molecule is evident. Measurements of the stability constant of Fe(III)-transferrin complex imply that the concentration of free Fe(III) ions in equilibrium with transferrin in blood is only about 10^{-24}M, thereby eliminating the problem of hydrolysis (Aasa et al.,1963; Aisen & Leibman,1968). The gross oversimplification of equation (5) is emphasized by the fact that no individual component of $Fe(OH)_3$ has actually been isolated (Durrant & Durrant,1970). A series of steps are thought to occur (Knight & Sylvia,1974) of which the following have been identified:

$$Fe(H_2O)_6^{3+} \rightleftharpoons Fe(H_2O)_5(OH)^{2+} + H^+ \quad (6)$$

$$Fe(H_2O)_5(OH)^{2+} \rightleftharpoons Fe(H_2O)_4(OH)_2^{+} + H^+ \quad (7)$$



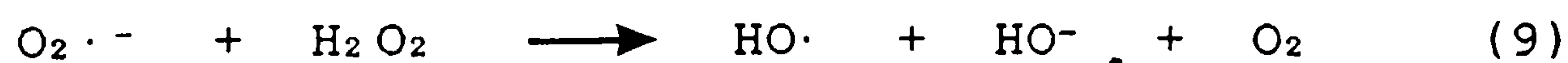
$$2Fe(H_2O)_5(OH)^{2+} \rightleftharpoons Fe_2(H_2O)_8(OH)_2^{4+} + 2H_2O \quad (8)$$

Some ligands will not necessarily protect against condensations eg, EDTA and nitriloacetic acid (Schuger *et al.*,1969).

1.9 Iron and free radicals

1.9.1 Fenton reaction

Since the Fe(II)/Fe(III) couple entails a one electron transfer, iron catalyses many redox reactions in which free radical intermediates are formed. A major question that arose in the late 1960's concerned the nature and origin of various deleterious effects observed when superoxide and hydrogen peroxide were present simultaneously in a biological system. The supression of these effects by the addition of catalase or superoxide dismutase or both led to the belief that HO· radicals were generated by the 'Haber-Weiss' reaction (Haber & Weiss,1934):

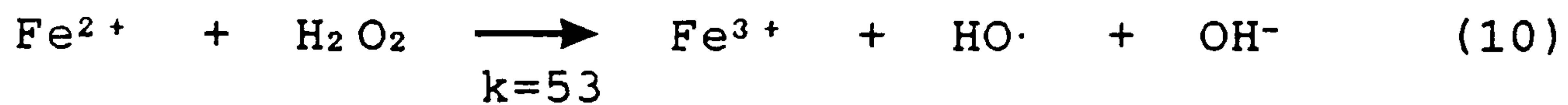


$$O_2^{\cdot -} + H_2O_2 \longrightarrow HO^{\cdot} + HO^{-} + O_2 \qquad (9)$$

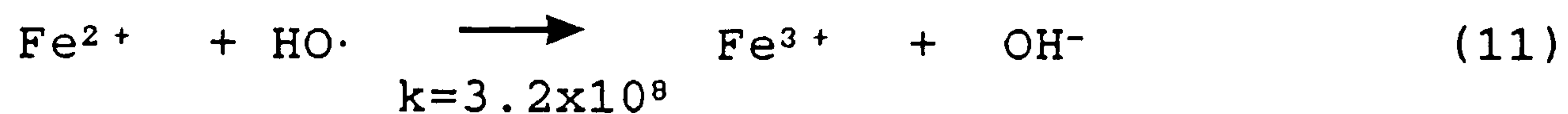
The reactivity between $O_2^{\cdot -}$ and H_2O_2 is reported to be very low (k values range from 10 to 10^{-4} $M^{-1}s^{-1}$, Weinstein & Bielski,1979). The reaction is thus unlikely to be an efficient source of HO· under physiological conditions. The consideration of the involvement of metal therefore became inevitable. After careful investigation of the system the overall mechanism was revised (Barb *et*

al. 1951; Bielski et al.,1985) by the following reactions:

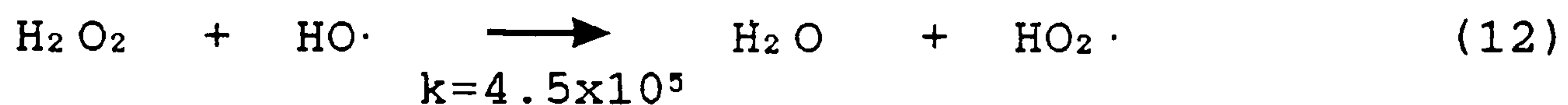
(k in $M^{-1}s^{-1}$)



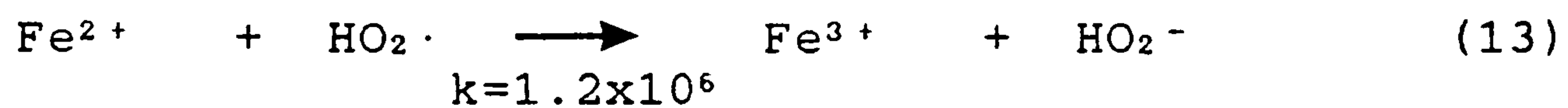
$$Fe^{2+} + H_2O_2 \xrightarrow[k=53]{} Fe^{3+} + HO\cdot + OH^- \quad (10)$$



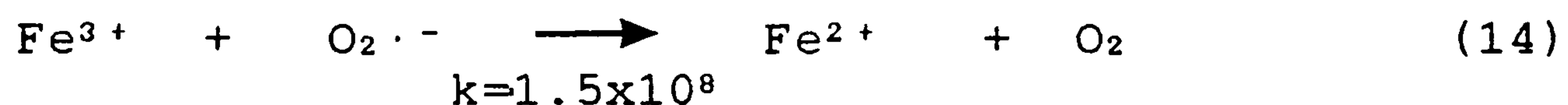
$$Fe^{2+} + HO\cdot \xrightarrow[k=3.2x10^8]{} Fe^{3+} + OH^- \quad (11)$$



$$H_2O_2 + HO\cdot \xrightarrow[k=4.5x10^5]{} H_2O + HO_2\cdot \quad (12)$$



$$Fe^{2+} + HO_2\cdot \xrightarrow[k=1.2x10^6]{} Fe^{3+} + HO_2^- \quad (13)$$



$$Fe^{3+} + O_2\cdot^- \xrightarrow[k=1.5x10^8]{} Fe^{2+} + O_2 \quad (14)$$

Thus, in a system containing excess H_2O_2, reaction (10) is the sole HO· free radical source in this system. This is the well known Fenton reaction (Fenton,1899). A mixture of Fe(II) and H_2O_2 (Fenton's reagent) itself is a potent oxidant in biological systems and will inactivate enzymes such as ribonuclease (Delgado & Slobodian,1972).

1.9.2 Tissue damage

Three important cases in which the contribution of a possible Fenton reaction may be largely responsible for tissue injury include (i) reperfusion injury, (ii) rheumatoid arthritis and (iii) iron overload.

(i) Tissue damage as a consequence of ischemia has by and large been attributed to the contribution made by oxygen metabolites that are generated from xanthine

oxidase at the time of reperfusion. Under non ischemic conditions the activity of xanthine oxidase in human tissue is low, but increases rapidly after the onset of ischemia. In a detailed review by Bulkley (1987), the mechanism of tissue injury involves the introduction of O_2 giving rise to a number of primary and secondary radicals in high concentration. Amongst this is the formation of H_2O_2 and HO· in the presence of reduced iron following electron transfer from superoxide. These radicals have been detected in reperfused myocardium using ESR (Garlick et al.,1987). Successful challenge with superoxide dismutase, catalase and DFO in the reperfused rat heart has led to the conclusion that free radical production is responsible for the damage caused in reperfusion (Davies et al.,1987a).

(ii) The respiratory burst of phagocytic cells inevitably increases the potential effects of oxygen radicals. The most striking consequences of this are seen in the autoimmune diseases (Halliwell,1982; Theofilopoulus & Dixon,1982) in which antibodies against normal body constituents are formed and provoke attack by phagocytic cells. Rheumatoid arthritis has many features of an autoimmune disease. Damage to the lubricating capacity of the synovium is due to breakdown of hyaluronic acid (Halliwell & Gutteridge,1984). This has been ascribed to the superoxide dependent formation of HO· upon activation by phagocytes (Greenwald & Moy,1980).

The neutrophils present in high concentration in synovial fluid contain micromolar quantities of iron salts (Blake _et al_.,1981a). A rapid fall in 'total iron' content of blood serum at the onset of inflammation is followed by a drop in Hb concentration in the synovial membranes (Blake _et al_.,1981b).

(iii) Studies of the pathology of iron overload point strongly to the involvement of the Fenton reaction (Bacon _et al_.,1983). The authors found a non-protein bound iron concentration of 26µM in the serum of one patient with idiopathic haemachromatosis. Patients with iron overload, and healthy Bantus, often have abnormally low contents af ascorbic acid in blood and tissues. Feeding them with ascorbate in the _absence_ of DFO has produced deleterious and sometimes lethal consequences, possibly because of increased lipid peroxidation and generation of HO· by ascorbate/iron salt mixtures (Nienhuis,1981).

Superoxide toxicity has often been ascribed to its ablility to reduce Fe(III) to Fe(II), consistent with its chemistry in aqueous solution. This suggestion has been criticised by several scientists, who argue that this might not happen _in vivo_, because the concentration of other biological reducing agents would be greater than $O_2{\cdot}^-$ (Fee,1982; Winterbourn,1981). Subsequent studies by Rowley & Halliwell (1982) and by Searle & Tomasi (1982) indicated that NADPH, NADH or thiol compounds could

indeed stimulate the production of HO· radicals *in vivo*. Ascorbate can under certain circumstances replace $O_2^{\cdot -}$ *in vivo*, especially where it is present at high concentrations such as eye (Varma *et al*.,1977) or pneumocytes (Castranova *et al*.,1983).

1.9.3 Alternatives to the HO· radical

In cases where the iron catalysed Haber-Weiss reaction cannot explain damaging effects of superoxide, because no protection is afforded by catalase or hydroxyl radical scavengers, the production of singlet O_2 has been proposed to cause the damage instead (Kellog & Fridovich,1977).

Alternative species to the hydroxyl radical have also been suggested by Walling (1982) who proposed the formation of a ferryl radical:

$$Fe^{2+} + H_2O_2 \longrightarrow FeOH^{3+} \text{ or } (FeO^{2+}) + OH^- \quad (15)$$

This radical has been suggested to be the reactive species at the active sites of horseradish peroxidase I and II (Dunford,1982) and of cytochrome P450 (Sligar *et al*.,1982). The existence of a perferryl radical, FeO^+ or $Fe^{3+}\text{-}O_2^-$ has also been raised, but its oxidising capacity is much lower than that of ferryl or HO· radicals (Tamura & Yamasaki,1972).

Critics have also questioned the feasability of production of "free" HO· radicals under conditions where

iron is bound to a ligand. Suggestions have been made by Aust _et al_. (1985), who have studied the effects of the relative proportions of Fe(II) and Fe(III) added to isolated microsomal preparations. These authors propose the existence of a highly reactive intermediate containing Fe(II) and Fe(III) simultaneously to which they attribute the effects of lipid peroxidation. By varying the relative concentrations of ascorbate (reductant) and DFO (an oxidant of ferrous iron), they obtained a threshold level of lipid peroxidation in the presence of iron, which declines if either reductant or oxidant concentration is exceeded. However, the degree to which this could be applicable _in vivo_ or to whole cells _in vitro_ is unclear and no evidence is available for the existence of such an intermediate.

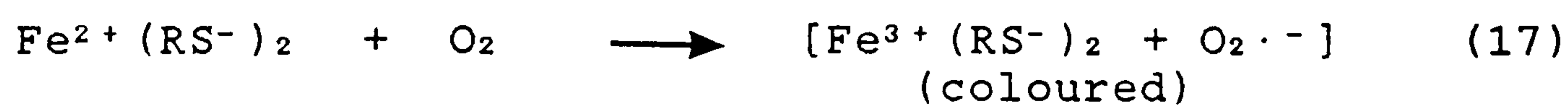
1.9.4 Reactions of iron with thiols

The importance of compartmentalisation on a molecular level has been stressed previously with respect to the damage caused by direct interaction of iron with thiols following excessive decompartmentalisation of iron (Willson,1977). This type of decompartmentalisation of iron might occur if one or more "compartment barriers" are destroyed, ie (i) a metabolic barrier; involving at least one species which is consumed, thereby preventing reaction of the species with its target molecule; (ii) a physical barrier; through which neither reactive species can diffuse and (iii) if both reactive species are

distantly separated by non-diffusible structures. The last 2 barriers are strikingly exemplified in the toxicity of encephalomyelitis virus. Rich in iron its action has been ascribed to the transport of iron across the blood-brain barrier which is normally impervious to the metal (Racker & Krimsky,1947).

In a simple aqueous solution, where these restrictions are absent the direct interaction of iron with thiols in the presence of the drug metronidazole or O_2 has been studied using stopped-flow techniques (Willson & Searle,1975; Willson,1977) and ESR techniques (Searle & Tomasi,1982). The kinetics of the appearance and disappearance of a red-purple colour are consistent with the one-electron transfer reaction with the oxygen-electron adduct, the superoxide radical anion, $O_2{\cdot}^-$:

$$2RS^- + Fe^{2+} \rightleftharpoons Fe^{2+}(RS^-)_2 \quad (16)$$



$$Fe^{2+}(RS^-)_2 + O_2 \longrightarrow [Fe^{3+}(RS^-)_2 + O_2{\cdot}^-] \text{ (coloured)} \quad (17)$$

1.10 Hydrogen Peroxide (H_2O_2)

1.10.1 Toxicity

H_2O_2 is a ubiquitous biological product formed as a normal enzymatic product of numerous oxidases present in the cell cytosol, plasma membranes, peroxisomes, mitochondrial matrix, mitochondrial outer membranes and blood plasma, as well as non functional (autoxidative)

reactions of haem proteins, flavoproteins and other cell components (Chance *et al*.,1979).

It is present in cells at concentrations as low as 10^{-8}M (Boveris & Chance,1973), but can be increased under certain conditions such as exposure to visible light (Parshad *et al*.,1978; Hoffmann & Menighini,1979), hyperbaric oxygen (Chance *et al*.,1979) and in the metabolism of certain carcinogens (Lorenzen & Ts'o,1977). H_2O_2 is also generated by monoamine oxidase-containing cells, by the electron transport chain and by phagocytes and may be involved in the mechanism of cell damage at sites of inflammation (Simon *et al*.,1981). Its production from superoxide by neutrophils functions primarily in the host's defence to assist in maintaining the body free from infection. However, when activated, the neutrophil may release superoxide and H_2O_2 into the extracellular space, and thus jeopardize the integrity of neighbouring cells. It is thought that the sudden burst of oxidants from neutrophils may overwhelm the protective antioxidant levels of endothelial cells which would lead to loss of integrity of the vascular component of the air:blood interface (Martin,1984). This has been associated with acute and chronic lung disorders.

The administration of H_2O_2 directly to combat various mouth disorders, advocated for many years as a useful mouth rinse, has led to serious side effects such as ulcerations and exacerbations of previously damaged

oral tissue as reported in 1986 by Rees & Orth.

1.10.2 Cytotoxicity

H_2O_2 is the relatively stable product of enzymatically catalysed dismutation of superoxide anions and has thus proved to be a useful experimental model for studying cell killing by O_2 intermediates (Rubin & Farber,1984). In these studies the direct effects were associated with lipid peroxidation and further work indicated additional damage which was independent of lipid peroxidation. Many investigators have focussed on DNA as a direct molecular target, with increasing attention. It has been possible to compare the type of lesion with those produced by radiation (Ward et al.,1985; Hoffmann et al.,1984; Welsh et al.,1985). Concentrations as low as 10μM are sufficient to produce reduction of colony formation (Hoffmann & Menighini,1979). The type of lesion responsible for cell death is still highly disputable (Wang et al.,1980; Ward et al.,1987; Olson,1988). In 1980 Meneghini & Hoffmann provided evidence that the effect in the cell is mediated by transition metal complexed to macromolecules in the nucleus.

In 1985 Ward et al suggested that the reaction of the hydroxyl radical was common to both radiation and exposure to H_2O_2. The cellular repair kinetics of the lesions were found to be similar (Ward et al.,1983; Bradly & Erickson,1981). These authors attributed the

damage to DNA double strand breaks (DSB). For ionising radiation the number of DSB necessary for cell killing has been shown to be 40 DSB per cell (Elkind & Redpath,1977). Ward et al (1985) has provided evidence that ionising radiation causes cell killing by the production of locally multiple damaged sites (LMDS) in DNA. These are produced in regions of high radical density present immediately after the occurrence of the energy deposition events. In the case of H_2O_2 treatment these are more difficult to detect, because at the ambient temperatures used, repair processes are thought to compete with the damage production processes so that any measured yield at any specific time is the resultant of this competition (Ward et al.,1987).

In line with this is the knowledge that high LET (linear energy transfer) radiation produces linear dose response relationships, whereas low LET radiation usually produces curvature in survival plots for cells (Thacker et al,1982; Adams,1987). This has been associated with the cell's capacity to repair some of the damage caused by radiation.

AIMS OF THIS THESIS

The work described in this thesis was undertaken to provide further support for the involvement of iron in the cytotoxic action of hydrogen peroxide. It was proposed to investigate the following main areas:

1. the properties of iron complexes by elucidation of some physico-chemical aspects of ligands which influence the reactions of iron with particular reference to iron dextran which may reflect some of the reported side effects associated with its administration. Emphasis is placed on comparitive studies with other iron complexes such as iron/EDTA or iron/ATP.
2. the cytotoxic action of hydrogen peroxide *in vitro* and manipulation of this cytotoxic effect by the addition of iron complexes;
3. the direct interaction of free radicals with a natural iron-binding ligand and its subsequent effect in an *in vitro* system.

CHAPTER II: CHEMICAL STUDIES ON THE REACTIONS OF IRON COMPLEXES AND HYDROGEN PEROXIDE

2.1 INTRODUCTION

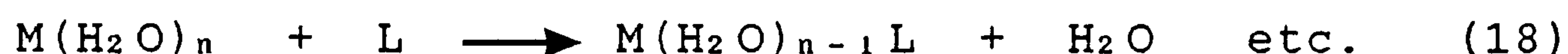
2.1.1. Effects of ligands on the chemistry of iron

The properties of iron in biological systems are largely affected by the compound to which it is bound. Very little metal exists in a free form in biological systems and reaction kinetics of unbound metals derived from simple chemical stoichiometry in aqueous solution must be viewed with caution. Factors governing the ability of a metal to interact with a ligand include the valency, size and charge of the metal and the dipole moment between metal and ligand. Another important point to consider is the electronic structure of the metal, which will be under influence of other electric fields, as well as the polarizability of the ligand.

As a prerequisite to examining the properties of bound iron the terminology used to describe the iron compounds is defined by the following phenomena:

1. Molecules with atoms coordinated to a metal ion are called ligands, based on the definition by Ringborn (1963).
2. The maximum number of coordinated atoms is the coordination number of the metal ion.
3. Ligands may be mono or polydentate according to the number of coordinating atoms that they contain.

4. Polydentate ligands are called chelating agents.
5. A metal ion coordinated by one (or more) ligand(s) is called a complex.
6. In general, specific complexes were prepared by adding an excess of a specific ligand to an aqueous solution of the metal salt. In aqueous solutions of metal salts, the metal ions are coordinated by water molecules. The hydration energy of a metal ion (M) is of the order of several hundred kilocalories, and reactions with other ligands (L) involve the replacement of one or more of the water molecules: eg.



$$M(H_2O)_n + L \longrightarrow M(H_2O)_{n-1}L + H_2O \quad \text{etc.} \qquad (18)$$

This terminology is used throughout the thesis to describe processes involving coordination complexes of iron.

Abbreviations

1. Complexes with specific ligands (L) are abbreviated in the following way;

Fe/L eg. Fe/EDTA, Fe/dex, Fe/ATP.

2. The number (n) of specific ligand molecules coordinated to the metal is indicated as $Fe(L)_n$ eg. $Fe(phen)_3$.
3. Where the redox state of the coordinated metal is known it is denoted by a superscript eg. $Fe^{2+}(FZ)_3$.

4. The most stable redox state of the metal ion in a specific complex in oxygenated solution is indicated as a Roman numeral in parenthesis eg. Fe(II)$(phen)_3$ or Fe(II)/ATP.

Some of the commonly used ligands are briefly described below;

2.1.2 EDTA (ethylenediaminetetraacetic acid)

One of the most widely used chelating agents is EDTA (fig.2.1). It chelates iron very well, but also binds other metals effectively. It interferes with iron absorption and is added to many foods as a preservative (Forth & Rummel,1973). With iron a hexadentate ligand is formed. Autoxidation of the Fe(II) complex is rapid (Jones & Long, 1952). In the ferric ((Fe(III)) form a water molecule is also co-ordinated to the metal in a structure which has been described as pentagonal bipyrimid by Hoard (1961). The complex forms five, five-membered rings, (thereby reducing the net charge on the metal ion).

2.1.3 DTPA (diethylenetriaminepentaacetic acid)

DTPA (fig. 2.2) is similar in structure to EDTA although more than one type of complex can be formed with iron with DTPA having 8 possible donor atoms. When all the coordination bonds are occupied the complex shows no physical constraint in contrast to the complex with EDTA. The structure of the complex depends on the pH of the solvent, but more than one possible form has been

STRUCTURE OF EDTA

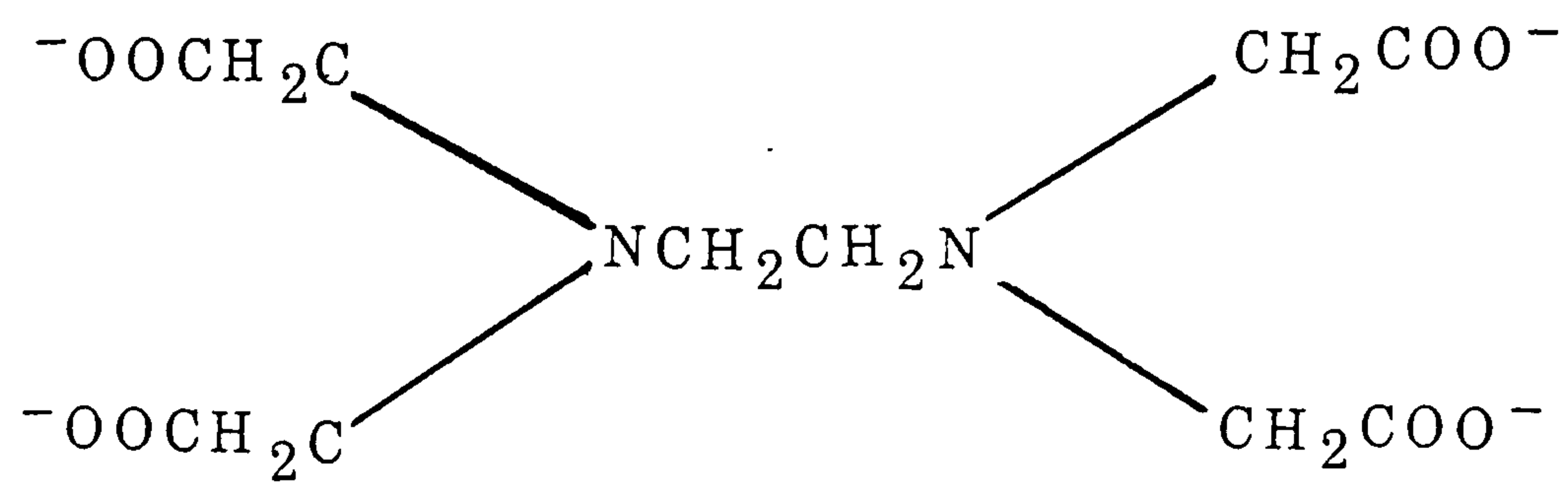


fig 2.1

STRUCTURE OF DTPA

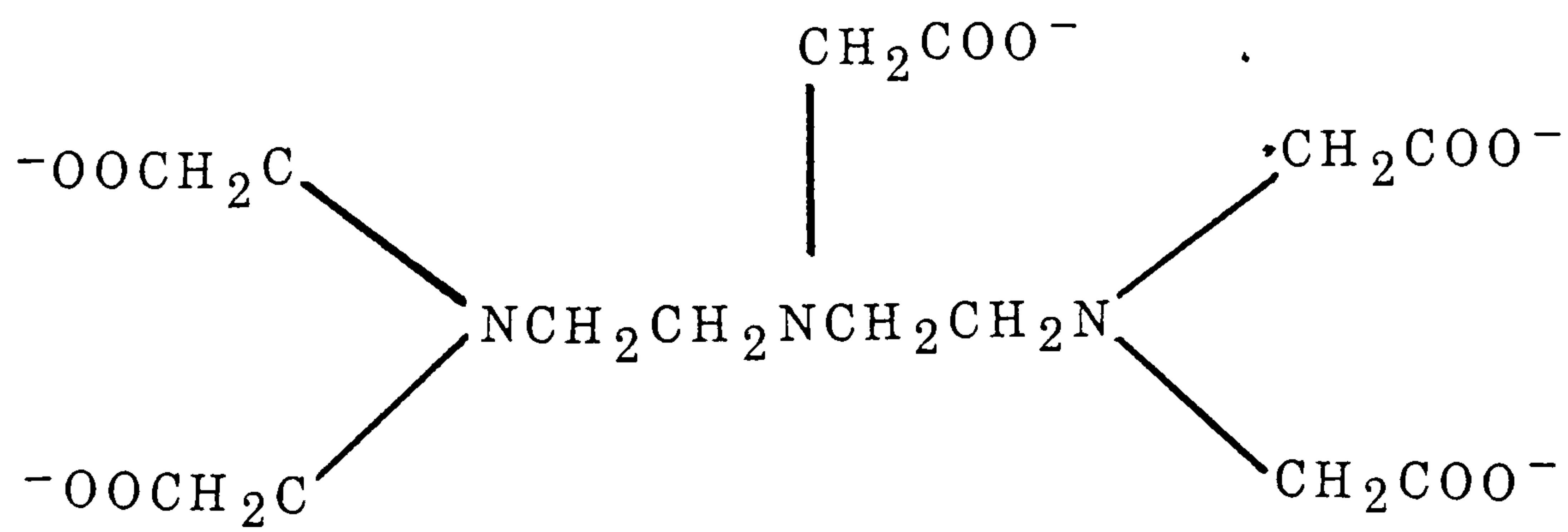


fig 2.2

suggested (Kostrimina,1974). Here too the Fe^{3+} complex is more stable than the Fe^{2+} complex.

The ability of DTPA to complex with metals is well known (Kroll et al. 1952). It is sometimes used to treat heavy metal poisoning and chlorosis (iron deficiency) in plants (Naukova, 1965).

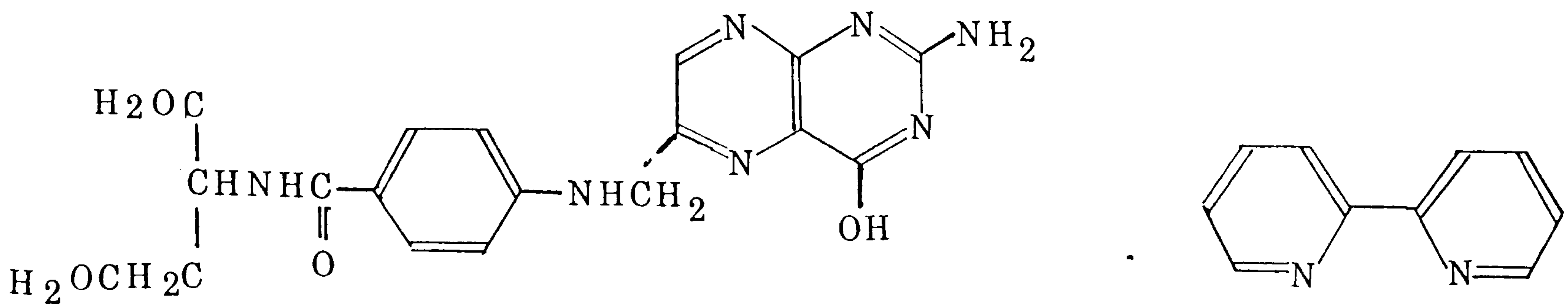
2.1.4 Heterocyclic nitrogen-type ligands

Only a few ligands are known to retain iron in the lower oxidation state. In particular these are the pyridine and pyrimidine type compounds which contain nitrogen capable of forming strongly coloured complexes with Fe^{2+} with consequent loss of paramagentism. Examples of these include bipyridyl, folic acid and o-phenanthroline (fig. 2.3) (Albert, 1986). These ligands are often used for spectrophotometric determination of Fe^{2+} (Bumbry & Massey, 1967; Beinert, 1978).

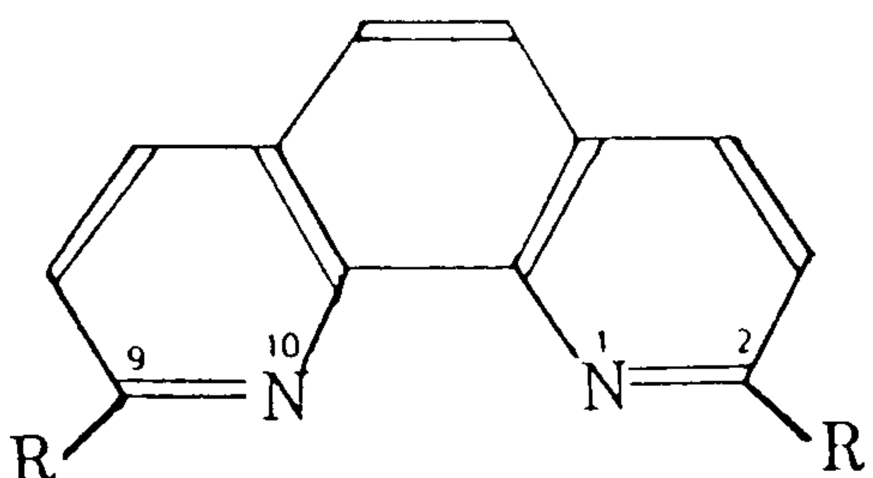
2.1.5 Ferrioxamines

Some of the strongest ligands for Fe^{3+} are the ferrioxamines. These hexapeptides are synthesised by a species of Streptomyces and X-ray diffraction studies have shown that the ferric ion is held by 6 oxygen atoms of three hydroxamic acid groups (Moshlin & Schnider,1963). Desferrioxamine, the most widely used chelator of this group, is described in Chapter I. It forms a bright red complex with Fe(III) with a binding constant of 31 (Albert,1986).

STRUCTURES OF SOME HETEROCYCLIC N-COMPOUNDS WHICH BIND IRON



FOLIC ACID



2,2'BIPYRIDYL

1,10-PHENANTHROLINE (R=H)

BATHOPHENANTHROLINE (R= ⌬)

fig 2.3

2.1.6 Iron dextrans

A widely used form of iron administration for iron deficiency are the iron dextrans. Interest in the potential deleterious effects of these compounds has arisen from reports of occasional unexplained side effects which are thought to be batch-related (Wenham, 1984).

Structural determinations of iron dextrans are based upon x-ray diffraction and electron microscopy studies. The complex is thought to be a colloid ferric oxyhydroxide (FeOOH) with the dextran acting as the stabilising hydrophilic colloid (Cox et al., 1971). No final structure has as yet been assigned to this complex. Alkali treatment of sugars substituted at C-6 results in the formation of metasaccharinic acid units (Kenner & Richards, 1956);

$$-CHOH.CHOH.CHOH.CH_2CHOH.CO_2H \qquad (V)$$

The final dextran has acidic end-groups and contains an average of 1 glucometascharinic acid residue per dextran chain. It has been suggested that this acid group protects the dextran against further attack by alkali (Bremner et al., 1969). The FeOOH core is attached to these terminal metasaccharinic acid units. The dextran chains are thought to radiate randomly from this core. Physical investigations carried out by Marshall &

Rutherford (1971) are indicative of a β-FeOOH form. This is distinct from the α form in that the OH groups in the β form are only weakly hydrogen bonded making it available to complex to carbohydrates (Gallagher & Phillips, 1969).

2.1.6.1 Manufacture of iron dextrans

The basic principles of Fe/dex (Imferon) manufacture have remained unchanged since 1954 and it was introduced by Fisons Pharmaceuticals. Other iron dextran complexes have been introduced (Imposil, Gleptoferron) for veterinary use with much lower ratios of dextran to iron but these have deviated little from the basic formula of Imferon processing. Imferon is an iron dextran complex made by reacting a soluble iron salt with alkali in the presence of a modified dextran containing carboxyl groups. The dextran fraction is made by a fermentation process which employs the enzyme dextran sucrase and converts sucrose to dextran and fructose. The high molecular weight dextran produced is harvested by solvent precipitation, hydrolysed and fractionated to remove unwanted high and low molecular weight species. The resultant product is treated with alkali (1M NaOH) to give a carboxylic acid derivative (dextran C) which is purified by solvent fractionation and can be isolated as a spray dried solid. The addition of ferric salt with alkali to dextran C produces a clear solution. Sodium chloride and sodium carbonate byproducts are removed by

precipitating the intermediate by decantation. The complex is redissolved in water at pH 6. The final solution is adjusted to contain 5% iron, filtered into suitable containers and sterilised by autoclaving (Wenham,1984).

2.1.7 Redox state of iron

The effect of ligands on the redox state of iron is probably one of the most important aspects from the biological point of view. In terms of damaging reactions the importance of oxidation in generating reactive oxygen species is clear. Many ligands are known to facilitate autoxidation of the complexed metal under various conditions. A list of examples is given below.

In this chapter some physico-chemical aspects of certain iron complexes are examined.

Ligands which facilitate $Fe^{(2+)}$ autoxidation	references
EDTA	Kurimura, 1968
nitrilotriacetic acid	"
tripolyphosohate	" 1969
amino acids	Astania, 1972
phosphoric acid	" 1971
oxalic acid	"
acetate	Grant, 1965
phosphoserine	"
phosphoethanolamine	"
citrate	Lee, 1969

2.2 MATERIALS AND METHODS

2.2.1 Solutions of iron salts

Ferrous sulphate ($FeSO_4$) and ferric chloride ($FeCl_3$) were obtained from SIGMA Chemicals Co.Ltd. Solutions were made up in single distilled water (and filter-sterilised before use with cells).

2.2.2 Solutions of ligands used for iron

The ligands o-phenanthroline, EDTA (ethylenediaminetetraacetic acid; disodium salt, dihydrate), DTPA (diethylenetriaminepentaacetic acid; free acid), citric acid, ADP (adenosine 5'diphosphate, sodium salt from yeast), ATP (adenosine 5'triphosphate, disodium salt, equine muscle), 8-hydroxyquinoline (8-HQ, free base), were obtained from SIGMA Chemical Co. Ltd. Ferrozine ([3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonic acid)-1,2,4-triazine disodium salt]) was obtained from BDH chemicals. Desferrioxamine (Desferal) was obtained from CIBA-GEIGY.

2.2.3 Preparation of iron complexes

Stock solutions of $FeSO_4$, $FeCl_3$ or complexing agents were made up as twice the required stock concentration and 20x the final concentration and dissolved in distilled water. o-Phenanthroline and 8-hydroxyquinoline were dissolved by gentle heating. DTPA was dissolved in alkaline conditions (5M NaOH) and then neutralised with 1M HCl. The iron complexes were then formed by mixing in a 1:1 ratio by volume. All the

complexes were prepared immediately prior to the experiment and the pH adjusted to 7.4 before making up to volume at 10x the final concentration with buffer. (The solutions were filter-sterilised for use with cells).

2.2.4 Iron dextrans

All batches of iron dextrans were obtained from Fisons Pharmaceuticals Plc. The batches, known as Imferon, contained 5% iron (50 mg/ml) and 20% dextrans (Sullivan,1985). Dilutions were made with distilled water. Dilutions were made to give a standard molarity of iron (100μM) with a corresponding dextran composition of 0.02%.

2.2.5 Buffer solutions

2.2.5.1 Phosphate buffered saline

Phosphate buffered saline was used as the incubation medium in most of the experiments. The composition was as follows: NaCl 8.0g; KCl 0.2g; $CaCl_2.2H_2O$ 0.132g; $MgCl.H_2O$ 0.1g; $Na_2HPO_4.2H_2O$ 1.15g; KH_2PO_4 0.2g; made up to 990 ml in distilled water. The pH was adjusted to 7.40 and the volume brought up to 1.0 litre. This solution was filter sterilised and is referred to in the following chapters as phosphate-buffered saline for incubation (PBSi).

2.2.5.2 Imidazole buffer

Imidazole-HCl was obtained from Sigma. The buffer (0.1M) was made up in single distilled water and the salts added in the same concentration as for PBSi (see

2.3.2.1). This was used as an alternative incubation buffer in experiments with the oxygen electrode involving iron-complexes.

2.2.6 Hydrogen peroxide solutions

Hydrogen peroxide, was obtained from Aldrich. The solution is 30% wt/v which corresponds to a concentration of 8.8M. Appropriate dilutions were made in single distilled water to give a 1 mM stock solution which was kept refrigerated for a maximum of 1 hour.

2.2.6.1 Assay for hydrogen peroxide: potassium iodide assay

The method for determining H_2O_2 was a chemical assay, based on that of Allen et.al., (1952) and involves the oxidation of potassium iodide by H_2O_2 or organic hydroperoxides in the presence of an ammonium molybdate catalyst. All reagents were purchased from Sigma. Two solutions were made up:

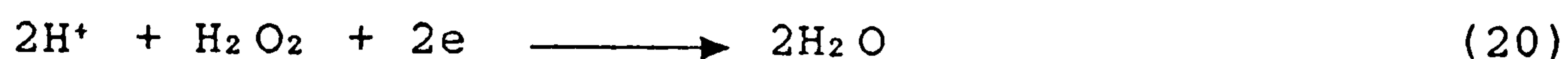
Solution A: 6.6g KI
0.2g NaOH
0.02g $(NH_4)_6Mo_7O_{24}.4H_2O$
in 100 ml distilled water

Solution B: 2g $HOOC.C_6H_4.COOK$
(potassium hydrogen phthalate)
in 100 ml distilled water

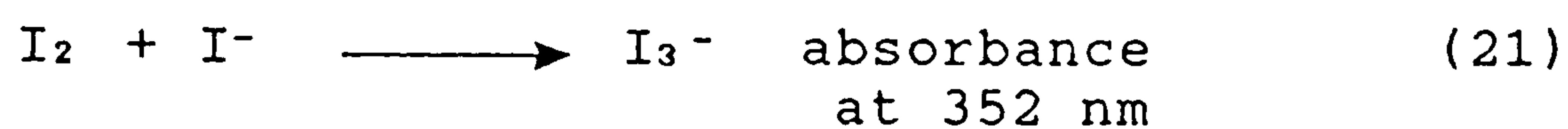
1 ml of each solution was added to two 1 cm path length cuvettes. Distilled water (0.5 mls) was added to the

control cuvette and 0.5 mls of the H_2O_2 solution to the test cuvette. Potassium hydrogen phtalate is used as a buffer, retaining a low pH to provide hydrogen ions for interaction with H_2O_2. The balanced reaction of KI with H_2O_2 iis given below:

$$2I^- \longrightarrow I_2 + 2e \qquad (19)$$



$$2H^+ + H_2O_2 + 2e \longrightarrow 2H_2O \qquad (20)$$



$$I_2 + I^- \longrightarrow I_3^- \text{ absorbance at 352 nm} \qquad (21)$$

The liberated iodine is determined spectrophotometrically as I_3^- at 352nm. A standard calibration curve was used for measurements of H_2O_2 (fig 2.4) This method provided a rapid determination of H_2O_2 prior to each experiment. The limitations pointed out by some critics of this method, such as the interference by other products which react with H_2O_2 were not relevant to the conditions of the estimations described here.

CALIBRATION CURVE FOR KI OXIDATION BY H_2O_2

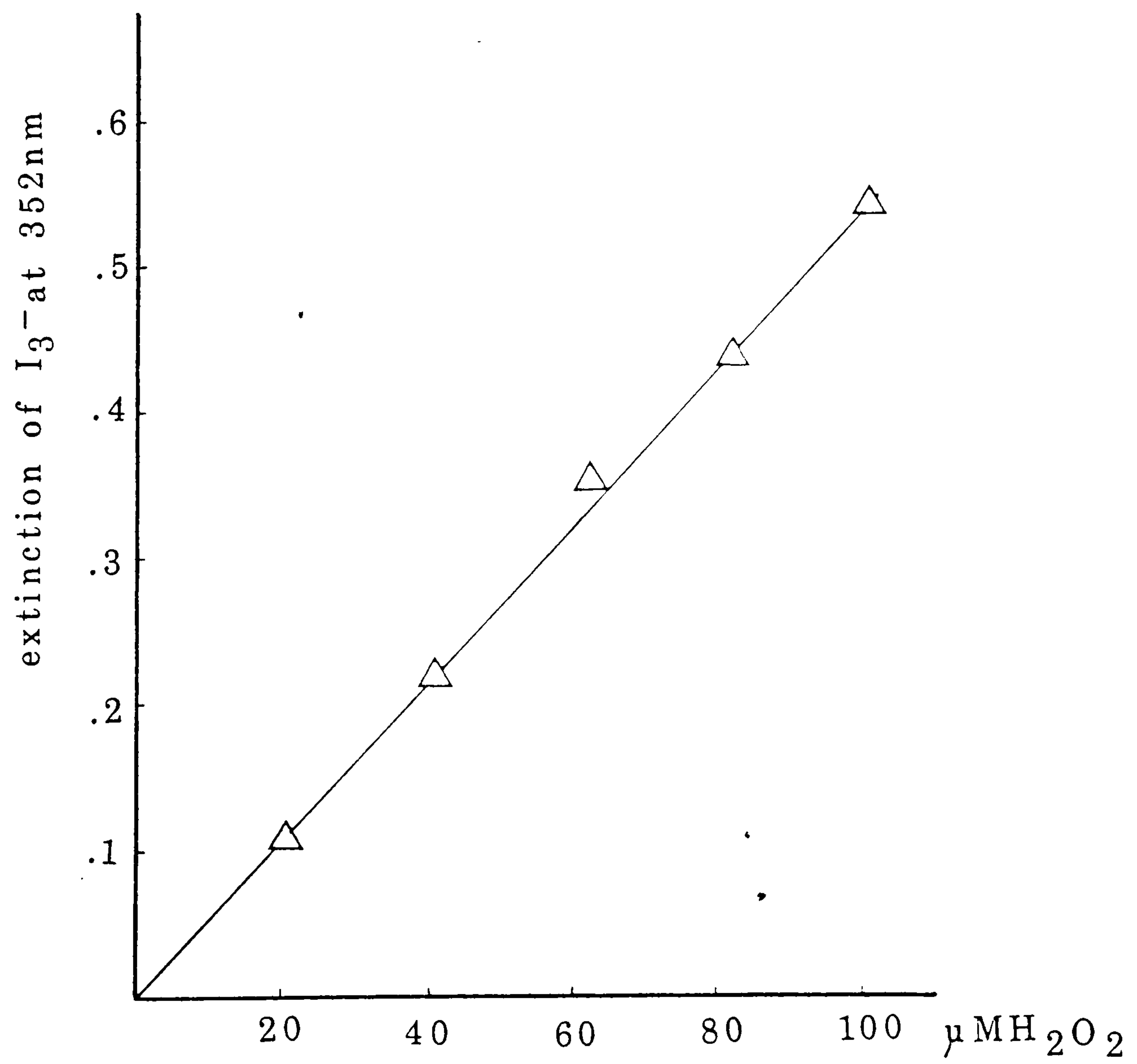


fig 2.4

2.3 RESULTS

A limited range of iron complexes was studied. Details of the preparation of these complexes are described in sections 2.2.1 to 2.2.4.

2.3.1 Partition coefficients

The relative solubility of iron complexes was assessed by their distribution between an aqueous and an imiscible organic phase. Solutions of complexing agents in the presence or absence of iron were prepared. The ratio of complexing agent to iron was 1:3. Each solution gave a characteristic absorption spectrum. The relative lipid solubility was estimated by the partition between water and petroleum ether (60-80° fraction). The aqueous solutions were shaken up with an equal volume of petroleum ether and the imiscible layers allowed to separate. The initial and final extinction of the aqueous phase at the peak absorption wavelength was measured for each compound and the partition coefficient (C) calculated as:

$$C = \frac{\text{Initial - final absorbance in aqueous phase}}{\text{final absorbance in aqueous phase}}$$

The partition coefficient of various ligands was estimated both in the absence of, and complexed to, iron in a ratio of 3:1. Table 2.1 gives the concentrations of the complexes remaining in the aqueous solution.

TABLE 2.1

PARTITION COEFFICIENTS OF IRON COMPLEXES

Agent (100µM Lig)[1]	Conc^n initial (µM)[2] in H_2O	Conc^n final (µM) in H_2O (after partition)	P/C.
ADP	100	100	0
ADP/Fe(II)	100	100	0
ATP	100	100	0
ATP/Fe(II)	100	100	0
Phen	100	100	0
Phen/Fe(II)	100	100	0
EDTA/Fe(III)	100	97	0.035
Fe/dex[3]	100	87	0.195
8-HQ	100	48	1
8-HQ/Fe(II)	100	49	1
8-HQ/Fe(III)	100	52	0.9

[1] In the presence of Fe the ratio of iron to ligand was 1:3.

[2] The concentrations refer to the concentration of ligand.

[3] Ratio Fe 5% : dex 20%

Lipid insolubility of Fe/EDTA is known. It is insoluble in a water-immiscible solvent (Albert,1986). The difference in solubility between Fe(II) and Fe(III)/8-HQ is negligible. Fe/dex shows a very small miscibility with the organic phase. Fe/dex has no disinct absorption peak; the absorption decreases steadily from 200-500nm. Following very slight partition into petroleum ether a reduced absorption spectrum was obtained which paralled the initial spectrum. A point at 300nm was chosen to calculate the partition coefficient.

2.3.2 Effect of ligands on the redox state of iron

The rate of oxidation of iron was found to be influenced by the nature of the complexing agent. Characteristics of the buffer were also investigated as some buffers used in biological systems are known to have ligand effects themselves which may affect the redox state of metals (Kobayashi et al.,1983; Saha & Sigel,1982).

The effects of ligands on the rate of oxidation of Fe^{2+} was studied by two methods; (i) measurement of oxygen consumption by an oxygen electrode, (ii) measurement of residual Fe^{2+} with time as measured by the formation of the complex between Fe^{2+} and ferrozine, a sensitive indicator of reduced iron.

In both assay systems the rate of Fe^{2+} autoxidation was compared as a function of the nature of ligand and related to published data from stability constants and

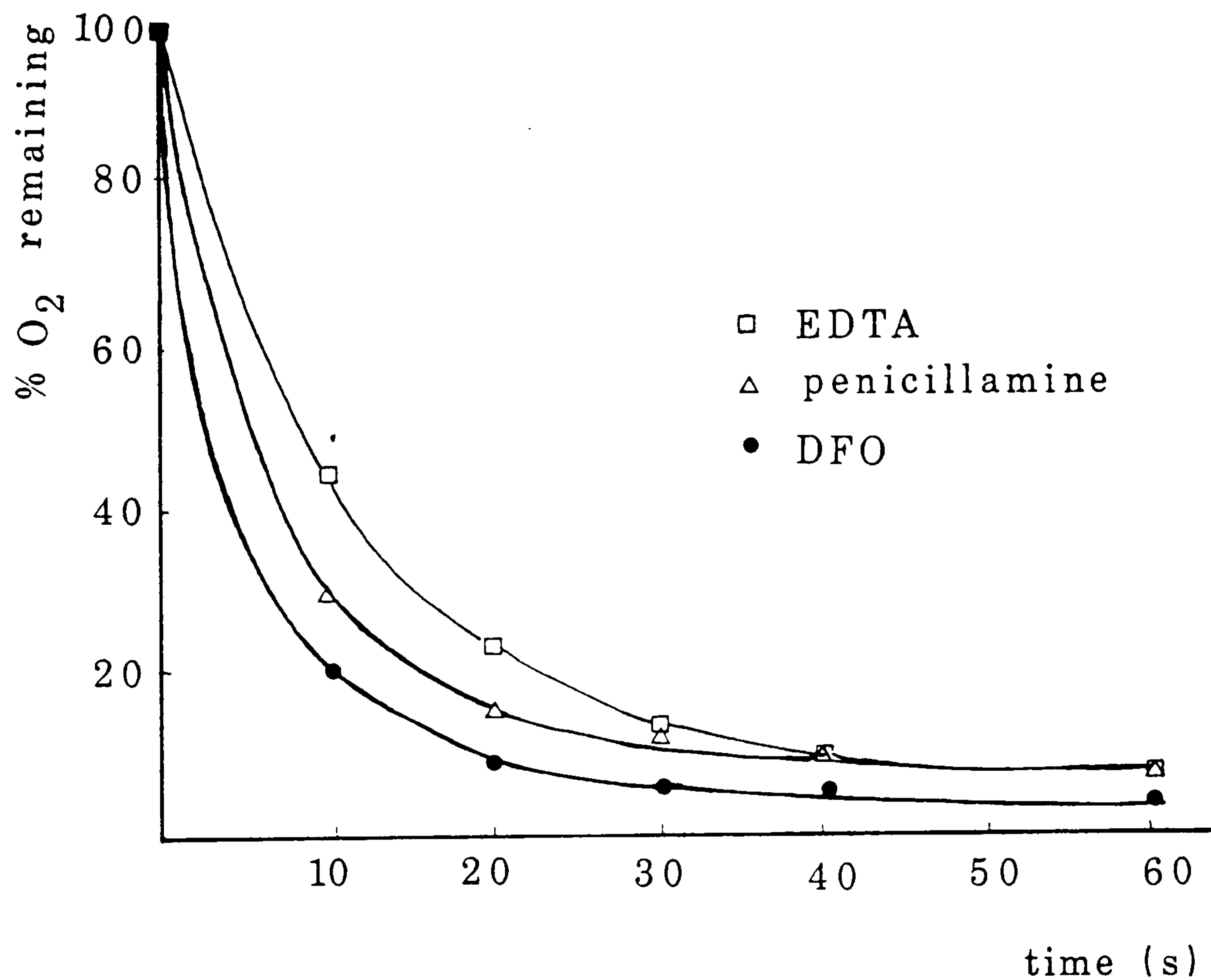
redox potentials.

2.3.2.1 Measurement of O_2 consumption by iron complexes

The rate of oxygen consumption by iron complexes was monitored using a (Clark type) O_2 electrode. The electrode was calibrated from 0 - 100% (200μM) saturation with O_2 by the addition of a small amount of dithionite. The chamber into which the electrode was placed contained a total of 1.8 mls. The initial concentrations of $FeSO_4$ and complex in the chamber were 1 mM and 5 mM respectively in 46 mM imidazole buffer at pH 7.40. The chamber was closed and the rate of O_2 utilisation measured at room temperature. The order of addition was buffer, followed by the ligand after which time the chamber was closed and a base line obtained. $FeSO_4$ was then added through a thin glass bore tube using a 50 μl Hamilton syringe. The complexing agents (ligands) investigated included: ATP, ADP, AMP, cAMP, L-histidine, glycine, penicillamine, EDTA, DFO, DTPA, phenanthroline and ferrozine. All compounds were dissolved in single distilled water. DTPA was prepared as described in section 2.2.3.

Fig 2.5 (a & b) show the oxygen consumption with time for various complexes and shows that over the time scale measured (60 mins) oxygen consumption by iron(II) bound to either phenanthroline or ferrozine is absent. Very rapid O_2 consumption occurs when iron is complexed to DFO, EDTA and penicillamine. Oxygen utilisation by iron

OXYGEN UTILISATION RATE BY $FeSO_4$ IN THE PRESENCE OF LIGANDS



Imidazole-HCl buffer 46mM pH 7.40

$FeSO_4$ 1mM

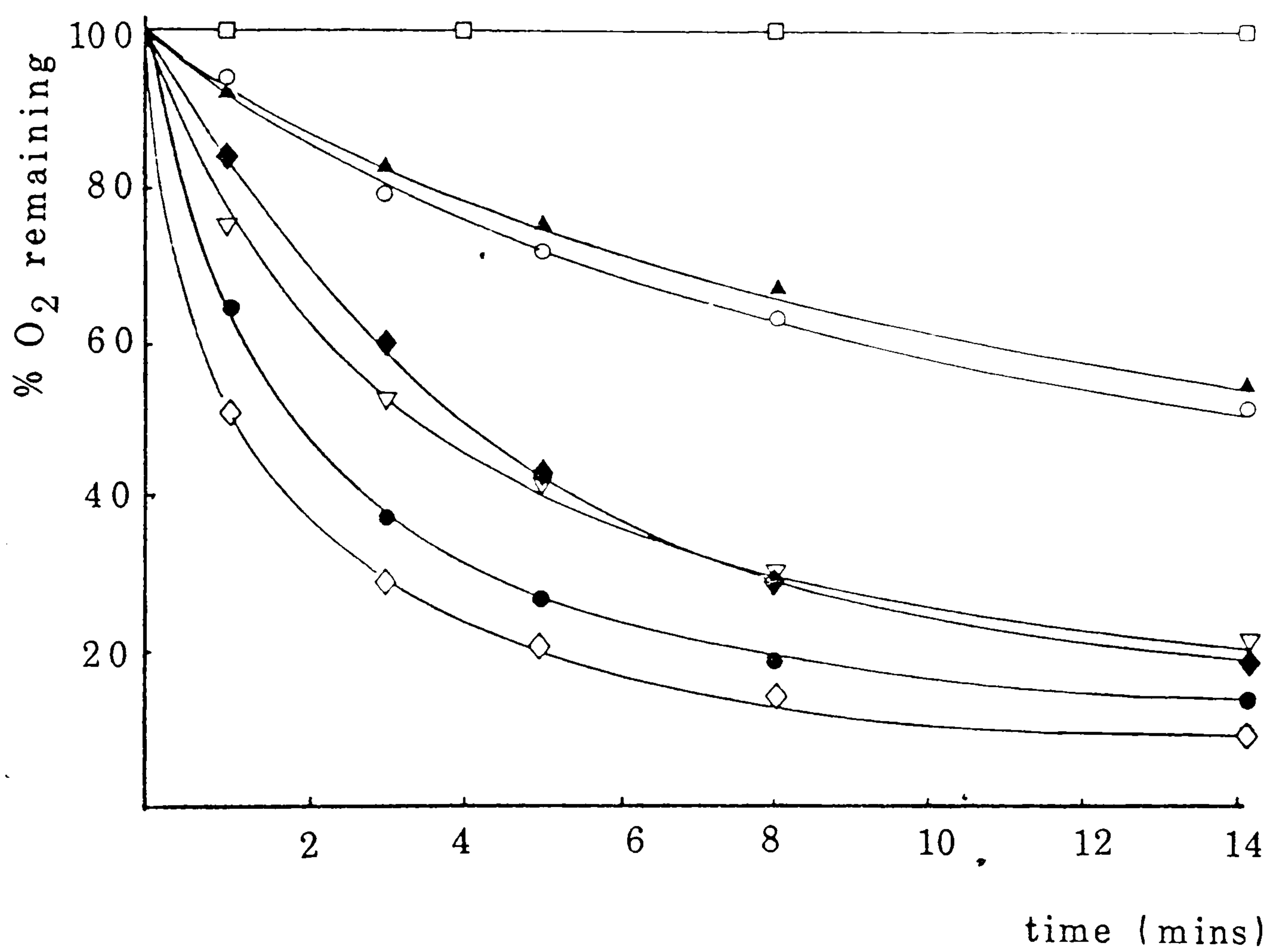
Ligands 5mM

fig 2.5 a

Imidazole-HCl buffer 46mM

$FeSO_4$ 1mM

Ligands 5mM



□ phenanthroline or ferrozine

▲ DTPA

○ ATP

▽ histidine

◆ Im alone

● AMP

◇ ADP

fig 2.5b

in the presence of L-histidine, AMP or ADP was similar to that observed in imidazole buffer alone. It is noteworthy that the difference between AMP and ADP is very small compared to the AMP and the ATP/iron complex.

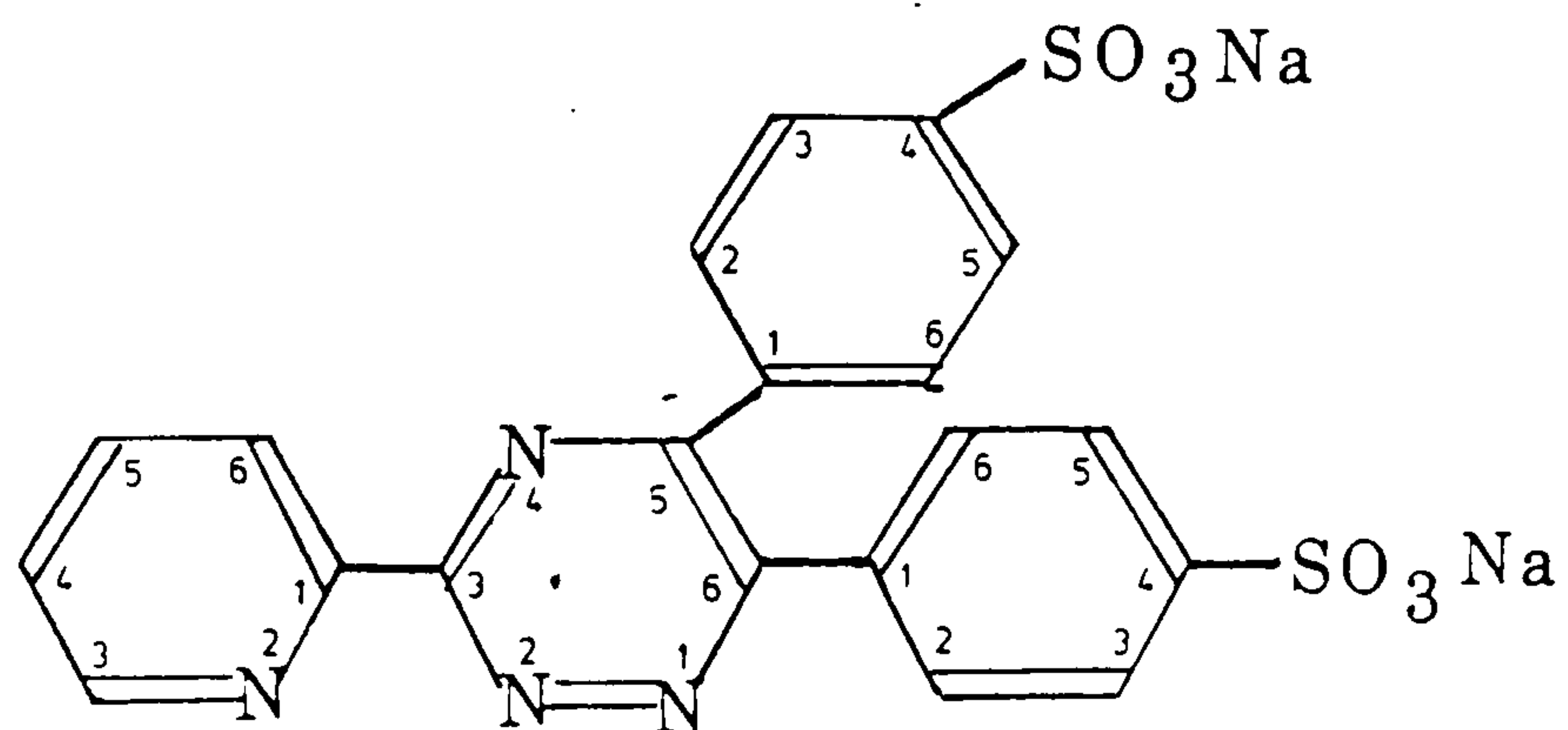
2.3.2.2 Ferrozine-iron(II) complex

The rate of oxidation of various iron(II) complexes was estimated using ferrozine. Ferrozine is a sensitive indicator of Fe(II) (Gibbs,1976; Anusiem & Gbemniyi,1978; Carter,1971; Langford et al.,1981; Adeniyi & Jaselshi,1980; Stookey,1970; Schilt & Hoyle,1967). It binds to Fe(II) in a ratio of 1 ion of Fe(II) to 3 molecules of ferrozine and the coloured complex formed has an extinction coefficient at 562nm of 28,000 $M^{-1}cm^{-1}$.

Ferrozine has the structure shown in fig 2.6. On the basis of its formula and spectral characteristics the $Fe^{2+}(FZ)_3$ complex probably has a structure similar to tris(2,2'-bipyridine)iron(II)(Schilt & Hoyle,1967). In this the three planar bidentate ligands are coordinated to the metal ion, each with two nitrogen atoms bound to form a five-membered ring, and with all six nitrogens in a an octahedral configuration about the central metal ion. In the case of ferrozine, the two most probable donor atoms for steric hindrance-free chelation are the pyridyl N-atom and the N-atom in position 2 of the triazine ring.

Electron delocalisation in the complex is pronounced resulting in an intense absorptivity of the

STRUCTURE OF FERROZINE



3-(2-PYRIDYL)5,6-BIS (4-PHENYLSULPHONIC ACID)-1,2,4 TRIAZINE

fig 2.6

complex. This suggests a planar conformation for each ligand to provide electronic conjugation among the rings. Sulphonation of the triazine groups ensure water solubility. The complex forms rapidly in the pH range of 4 to 9 (Stookey,1970).

A calibration curve was constructed for the absorbance of solutions containing increasing concentrations of $FeSO_4$ in distilled water. Fig 2.7 shows a standard calibration curve for the Fe^{2+}-ferrozine complex in the concentration range 0-84 µM $FeSO_4$. The saturation plot shown in fig 2.8 illustrates the effect of increasing ferrozine concentration with constant $FeSO_4$ concentration. $FeSO_4$ was used at a concentration of 100 µM. Saturation begins at 3.2×10^{-4} M of ferrozine, which is with a Fe^{2+} to ferrozine ratio of about 1:3 and agrees with published data (Gibbs, 1976). Consequently ferrozine was kept in excess of a molar ratio of 1:3 for all estimations of Fe(II) to ensure that complexation to ferrozine goes to completion in the presence of other competing iron ligands and the imidazole buffer.

Ferrozine was used to estimate the amount of Fe(II) remaining when iron was bound to different ligands. The ligand and $FeSO_4$ concentrations were 150 µM and 50 µM respectively. The buffer used was imidazole-HCl (10 mM) at pH 7.40. Measurements were made at room temperature (ca. 24°C). The solutions were measured at specific time intervals by the addition of 300 µM of ferrozine reagent.

CALIBRATION CURVE FOR FERROZINE WITH $FeSO_4$ IN WATER

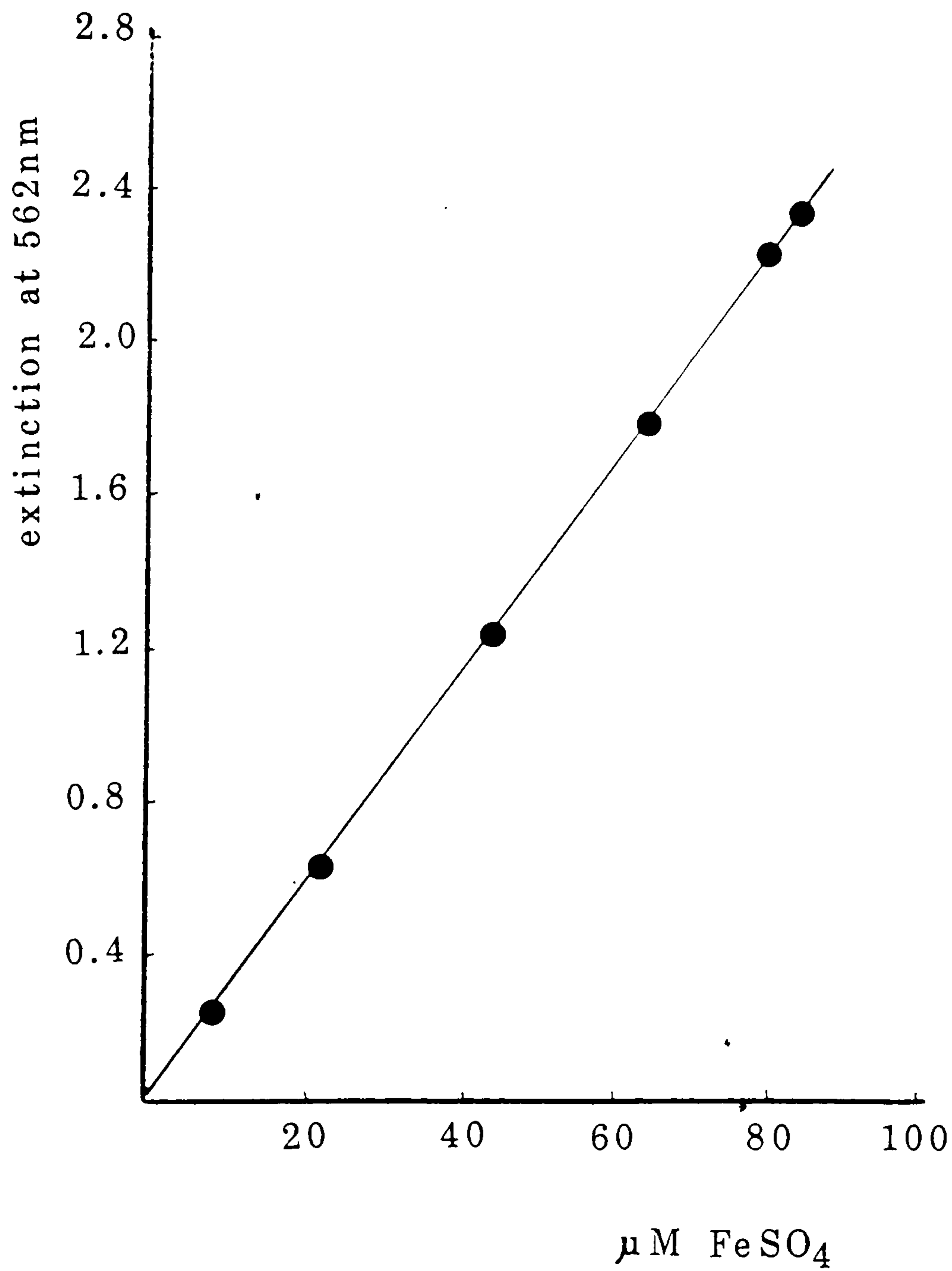


fig 2.7

SATURATION CURVE FOR FERROZINE WITH

100μM $FeSO_4$

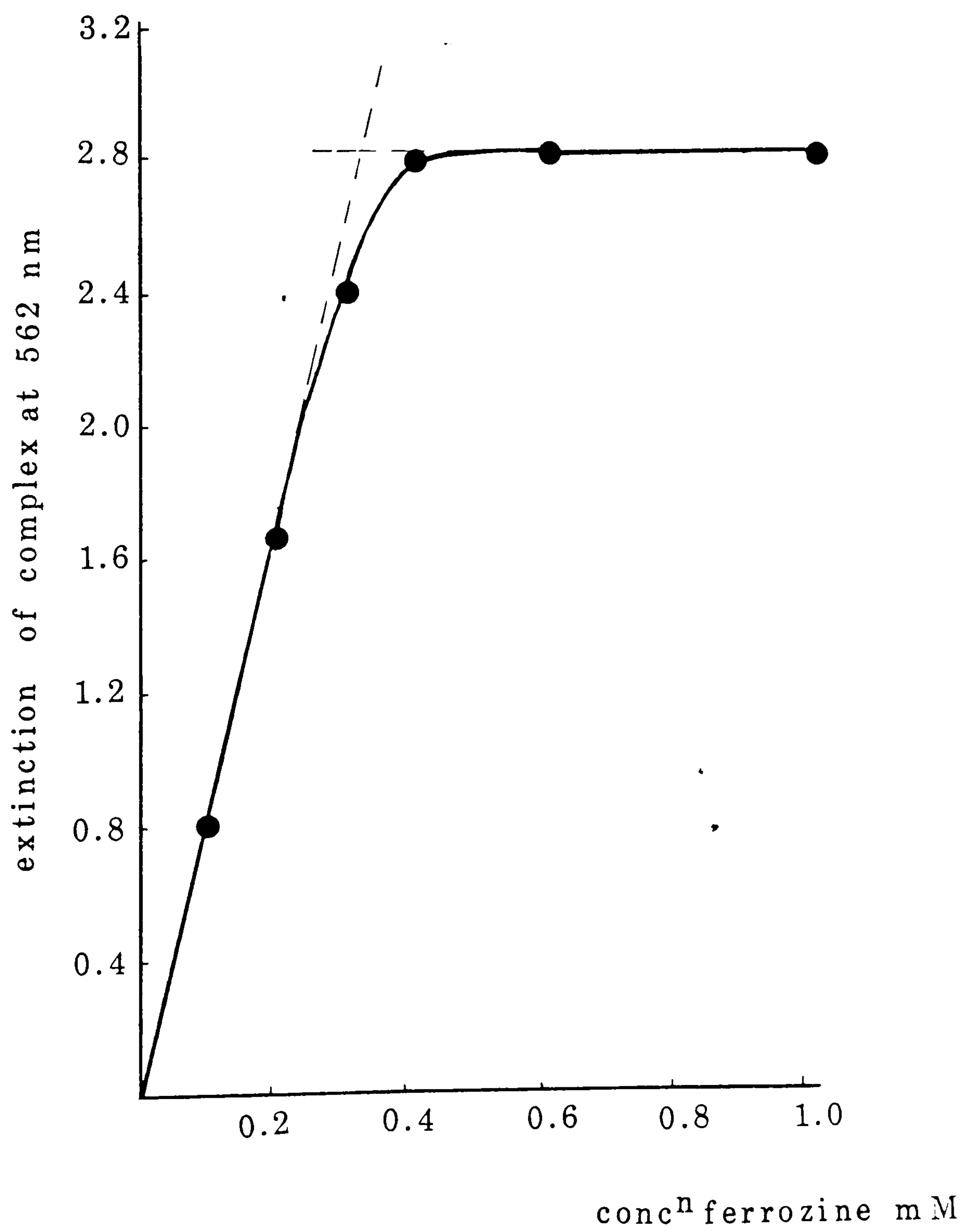


fig 2.8

The solution was well mixed (Vortex mixer) and allowed to incubate at 37°C for 30 minutes before the absorbance of the Fe(II)-ferrozine complex was measured.

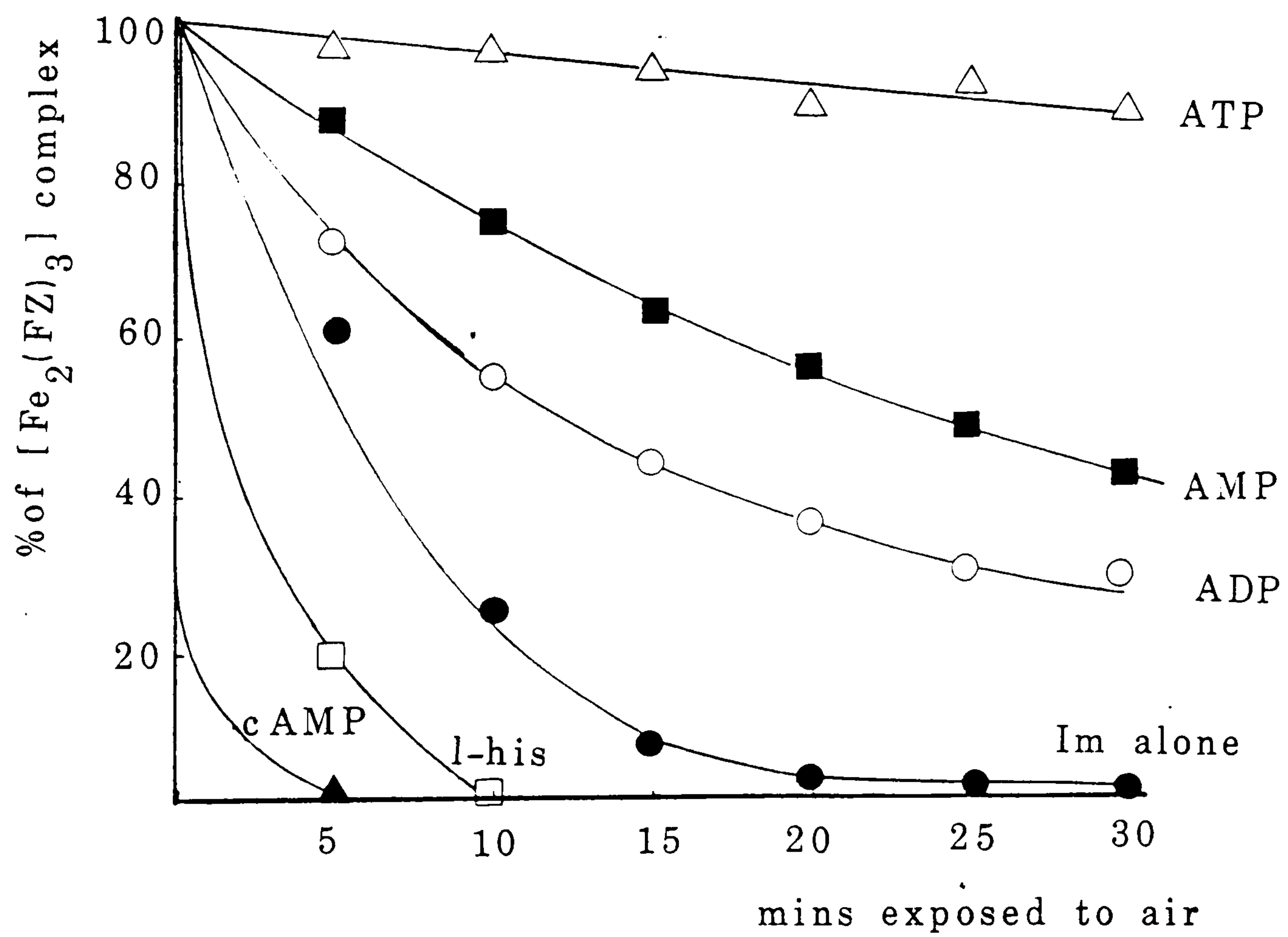
For comparison with the consumption of O_2 by the iron complexes the oxidation of Fe(II) to Fe(III) was indirectly measured by the amount of ferrozine complexable Fe(II) remaining as a function of time. The effect of some of the complexes tested is shown in fig 2.9.

Taking into account the different conditions in the experimental systems the data in fig 2.9 are in good agreement with those in fig 2.5(b).

<u>2.3.2.3 Comparison of the oxidation rates from both assays</u>

The rates of reaction between $FeSO_4$ and O_2 were calculated for the different complexes used. From figs. 2.5 and 2.9 the half life of Fe^{2+} autoxidation can be estimated provided the rates are exponential. Attempts were made to correlate the data between the two assays by using rate constants for each complex as obtained from the half life of either O_2 consumption or Fe^{2+} utilisation. However, the conditions of the two assays were sufficiently different to result in quantitative differences in the estimated rates. The O_2 concentration in the ferrozine assay is constant whereas it drops in the closed system used for in measurements made with the oxygen electrode. The rates of Fe^{2+} autoxidation as

RATE OF OXIDATION OF Fe^{2+} BOUND TO DIFFERENT LIGANDS



Im buffer 46mM pH 7.40

$FeSO_4$ 50µM

ligand 150uM

ferrozine 300µM

fig 2.9

assessed by these 2 methods are given in table 2.2.

2.3.2.4 Comparison of uncomplexed and complexed Fe(II) by phenanthroline or ferrozine

Because Fe(II) complexed to one ligand may not readily react with a second ligand even if it is present in excess it was considered prudent to examine the quantitation by ferrozine by use of another ligand as a 'detector' of Fe(II). The agent employed was o-phenanthroline instead of ferrozine. Both ligands form a 3:1 complex with Fe(II), but have different binding constants. Fe(II) was estimated in the μM concentration range either alone or complexed to ADP or dextran. Phenanthroline or ferrozine was added and the solutions left to equilibrate for 1 hour at room temperature before measuring the absorbance. All measurements were made in duplicate. All solutions were prepared in distilled water.

Table 2.3 shows the mean concentration of Fe(II) as $FeSO_4$ in distilled water, pH 5-6. Both phenanthroline and ferrozine give the same concentrations of Fe^{2+}. Similar results (table 2.4) between the two assays were also obtained when iron was complexed to ADP. In this experiment the ligand was present in a molar ratio of 20:1.

In this case the concentration of Fe(II) was lower than the initial concentrations, suggesting that under the conditions of the experiment ADP may permit

TABLE 2.2

RATES OF AUTOXIDATION OF IRON COMPLEXES

Complex[1] 1mMFe:5mM Lig	rate of O_2 utilisation M min^{-1}	rate of Fe^{2+} oxidation[2] M min^{-1}
$FeSO_4$[3]	4.6×10^{-8}	3.4×10^{-7}
Fe/histidine	5.8×10^{-8}	1.9×10^{-6}
Fe/AMP	1.1×10^{-7}	8×10^{-8}
Fe/ADP	1.6×10^{-7}	1.7×10^{-7}

[1] in imidazole buffer, 46mM, pH 7.4

[2] measured by the amount of ferrozine-complexable Fe^{2+} remaining after exposure to air for different times.

[3] freshly prepared $FeSO_4$ in imidazole buffer

TABLE 2.3

Fe^{2+} DETECTION BY PHENANTHROLINE OR FERROZINE

Initial $[Fe^{2+}]$* (μM)	mean $[Fe^{2+}(phen)_3]$ (μM)	mean $[Fe^{2+}(FZ)_3]$ (μM)
0	0.3	0.25
20	19	20
40	38	40
60	60	63
80	80	80

*Fe^{2+} represents a solution of freshly prepared $FeSO_4$ in distilled water with a pH of 5-6.

TABLE 2.4

Fe^{2+} DETECTION BY PHENANTHROLINE OR FERROZINE IN Fe(II)/ADP (1:20 COMPLEX)

Initial Fe(II)* (μM)	mean $[Fe^{2+}(phen)_3]$ (μM)	mean $[Fe^{2+}(FZ)_3]$ (μM)
20	19	19
40	38	39
60	55	56
80	74	75

* Fe(II) represents the concentration of a freshly prepared solution containing $FeSO_4$ and ADP in distilled water pH 5-6.

autoxidation of Fe^{2+} to Fe^{3+}.

In table 2.5 the comparable assays are shown in the presence of Fe/dextran which was previously reduced by heating with an excess of ascorbate.

A slight overestimation in the final iron concentrations may have occurred due to problems in the dilution of highly viscous Fe/dextrans.

2.3.2.5 Autoxidation of Fe/dextrans as measured by ferrozine

Fe/dextrans, exposed to air for varying lengths of time were measured for their Fe^{2+} content with ferrozine. Time intervals lasted up to 3 months. Different batches were tested as shown in table 2.6. For one particular batch (8K) the Fe^{2+} content was monitored for different times. Fig 2.10 shows the slow oxidation. The initial concentration of Fe^{2+} was 29 mM based on an average concentration of 0.9M total iron in these Fe/dextrans (Sullivan,1985). This agrees in order of magnitude with estimations made by Cox et al. (1965). Using bathophenanthroline they estimated between 9-18 mM Fe^{2+} for freshly openend Fe/dextrans.

2.3.2.6 Reduction of Fe/dex by ascorbate

Addition of ascorbate to Fe/dex leads to a slow reduction of the iron, which can be monitored by addition of ferrozine at different time intervals. This is in contrast to other Fe/complexes, in which the reduction is almost instantaneous. In fig 2.11 the rate of reduction

TABLE 2.5

DETECTION OF IRON AS Fe(II) IN ASCORBATE-REDUCED Fe/DEXTRAN BY PHENANTHROLINE OR FERROZINE

Initial [Fe] (μM)	mean $[Fe^{2+}(phen)_3]$ (μM)*	mean $[Fe^{2+}(FZ)_3]$ (μM)*
9	9	11
18	17	19
36	36	38
72	75	73
90	93	83

* mean concentration of Fe^{2+}/complex from duplicate samples.

Solutions were prepared in distilled water.

Solutions of Fe/dextran were heated to 60°C for 1 hour with excess ascorbate, allowed to cool and measured for Fe^{2+} content.

TABLE 2.6

VARIATION OF Fe(II) CONTENT IN IRON DEXTRAN BATCHES EXPOSED TO AIR FOR DIFFERENT TIMES

Batch	time from sealed ampoule	concentration of $Fe^{2+}(FZ)_3$ complex (μM)*	percentage of total Fe in dex %
3H	freshly opened under N_2	39	4.3
16H	1 month	15	1.7
8K	2 months	13	1.4
9L	3 months	10	0.9

* Ampoules were initially diluted 1/1000, then measued by ferrozine method. The total iron concentration in these dextrans is 0.9M or 5% (Sullivan, 1985)

Legend to fig 2.10:

Fresh ampoules were diluted to 10^{-3} M equivalent iron. Ferrozine (300μM) was added at various time intervals after exposure to air and samples left to equilibrate. O.D. measured at 532nm. Fe/dex batch 8K.

MEASUREMENT OF Fe(II)/FERROZINE FROM Fe/DEXTRAN EXPOSED TO AIR FOR DIFFERENT TIMES

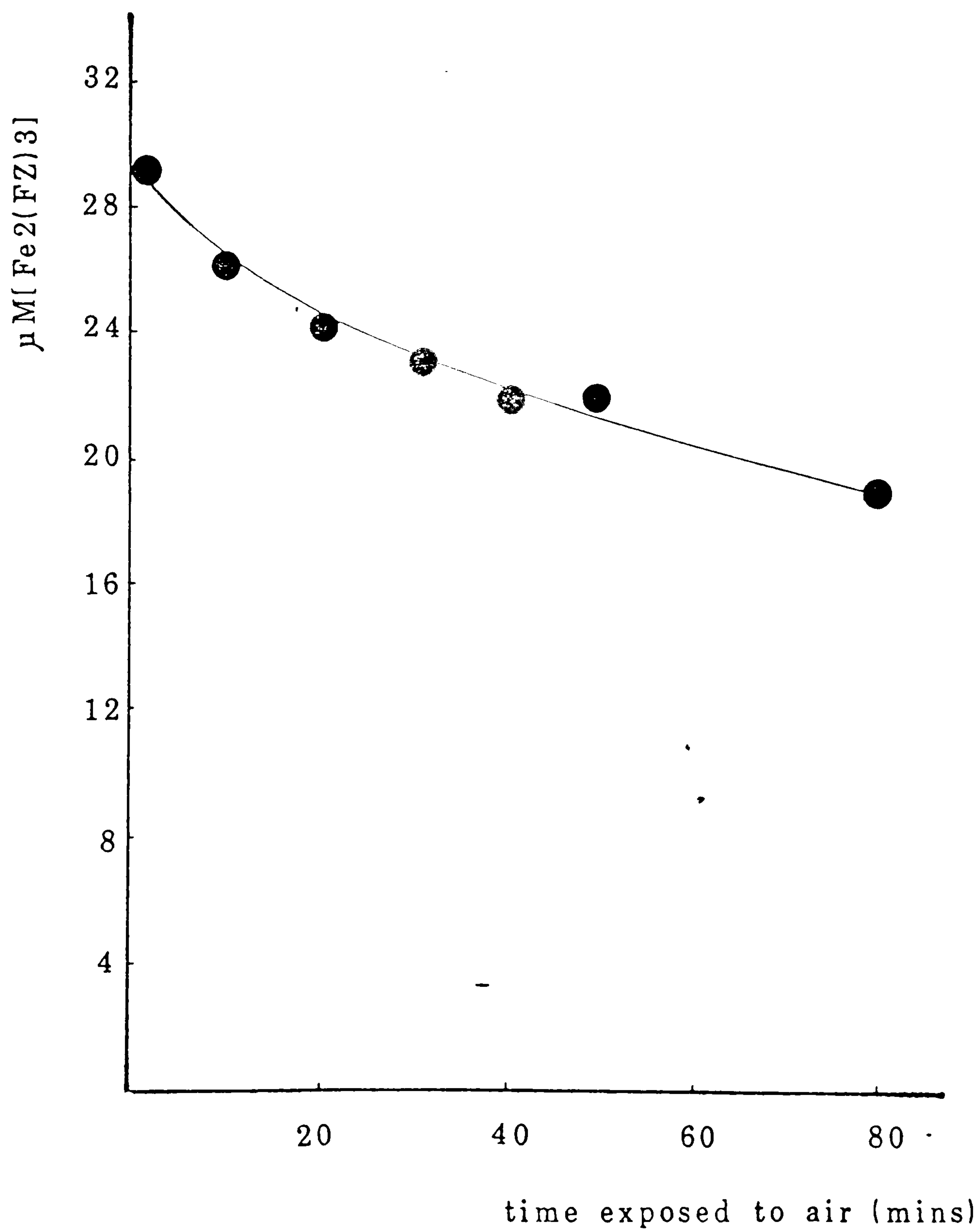
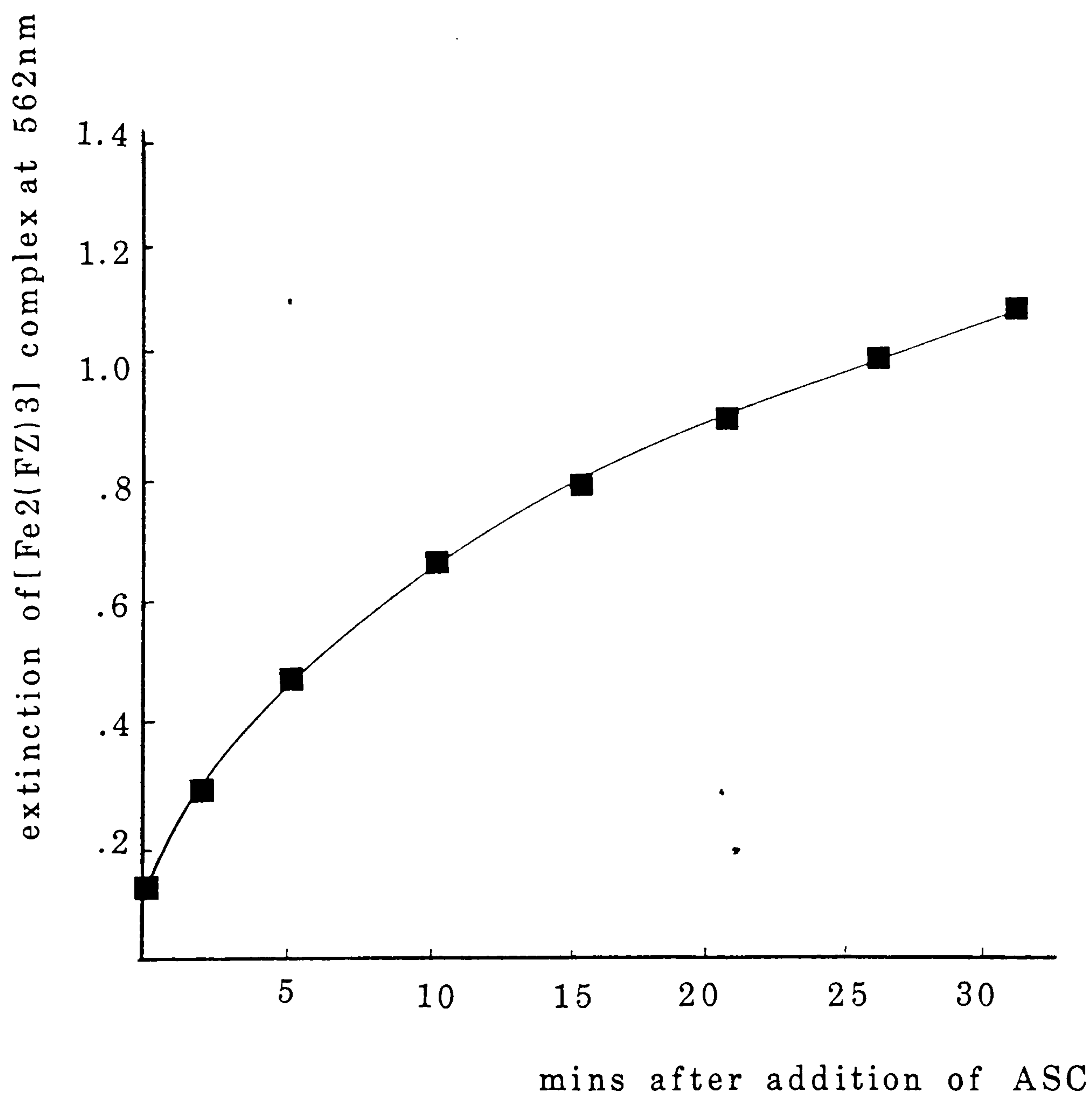


fig 2.10

RATE OF FORMATION OF Fe/FERROZINE COMPLEX FROM Fe/DEXTRAN IN THE PRESENCE OF ASCORBATE



Fe/dex 1mM

ASC 1mM

fig 2.11

to Fe^{2+} was measured as a function of the time of exposure to 1 mM ascorbate. When 1 mM ascorbate was added to 1 mM Fe/dex the initial rate of Fe^{2+} production was 3.25 μM/minute. The total amount of Fe^{2+} produced over 30 minutes was 35 μM. When Fe/dextrans were heated with ascorbate (in excess, 60°C) the rate of reduction was considerably increased. After 30 minutes of heating most of the iron was reduced; 47 μM Fe^{2+} was obtained from an initial iron concentration of 50 μM in the Fe/dex (fig 2.12) and the dextran solution turned colourless. In chapter IV, section 4.3.3.7 the effect of pre-heated Fe/dex with ascorbate on the cytotoxicity of H_2O_2 at 4°C is described.

2.3.2.7 Effect of ascorbate on the rate of autoxidation of Fe/EDTA

The rate of autoxidation of Fe/EDTA in the presence of ascorbate was studied. The chamber contained phosphate buffer (PO_4^{2-}concentration: 8mM) and the additions made as follows: 100 μM Fe(III)/EDTA (1:1) followed by 5 mM ascorbate.

In the presence of 5 mM ascorbate the half-life of O_2 was 2 minutes for a 1:1 ratio of $FeCl_3$ to EDTA (100 μM in PBSi) (fig 2.13).

2.3.2.8 Effect of ascorbate and H_2O_2 on the rate of O_2 consumption

The effect of addition of both oxidant and reductant to iron complexes was monitored by the rate of

EFFECT OF PRE-HEATING Fe/DEXTRAN WITH ASCORBATE ON THE FORMATION OF THE Fe/FERROZINE COMPLEX

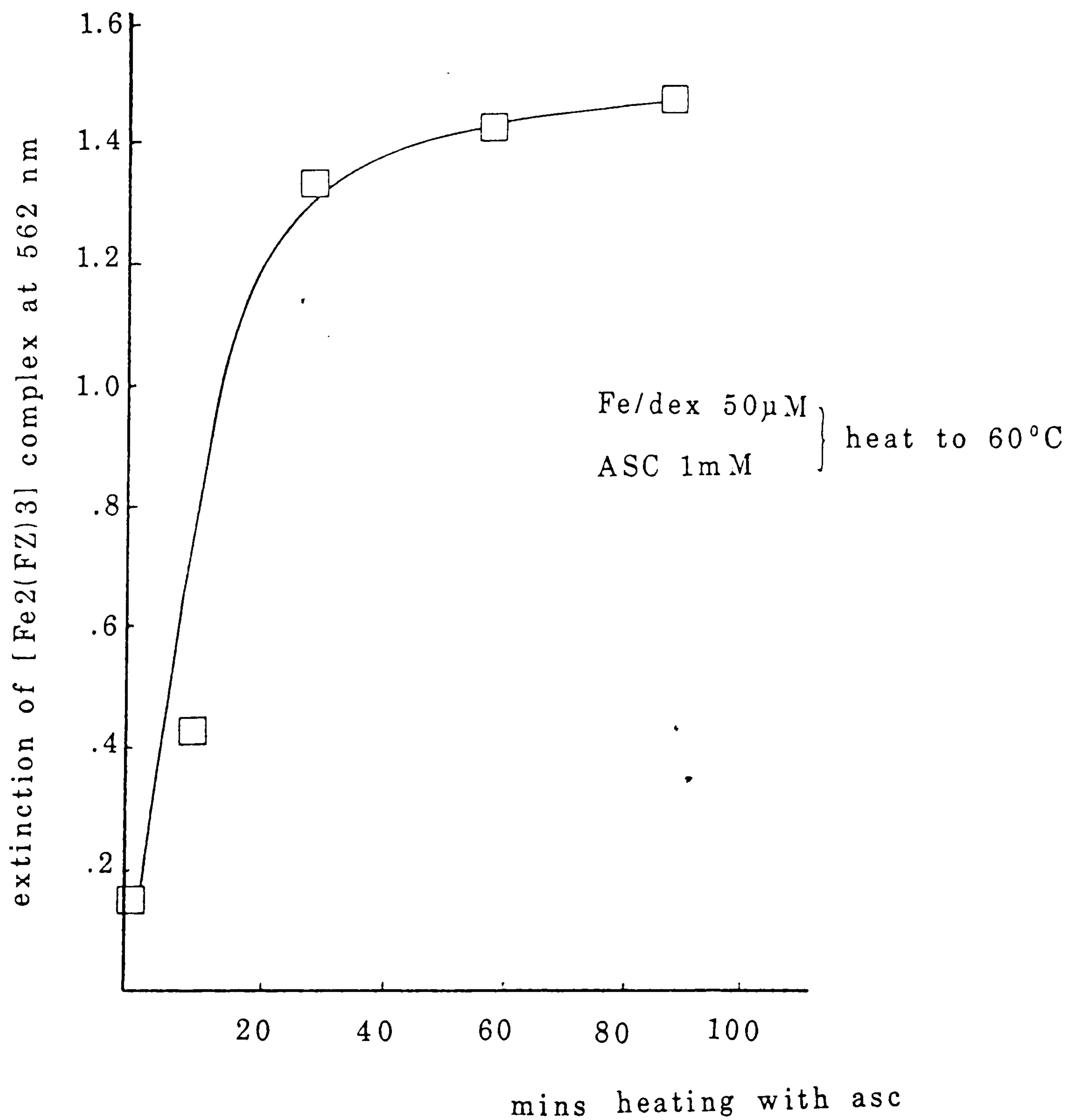
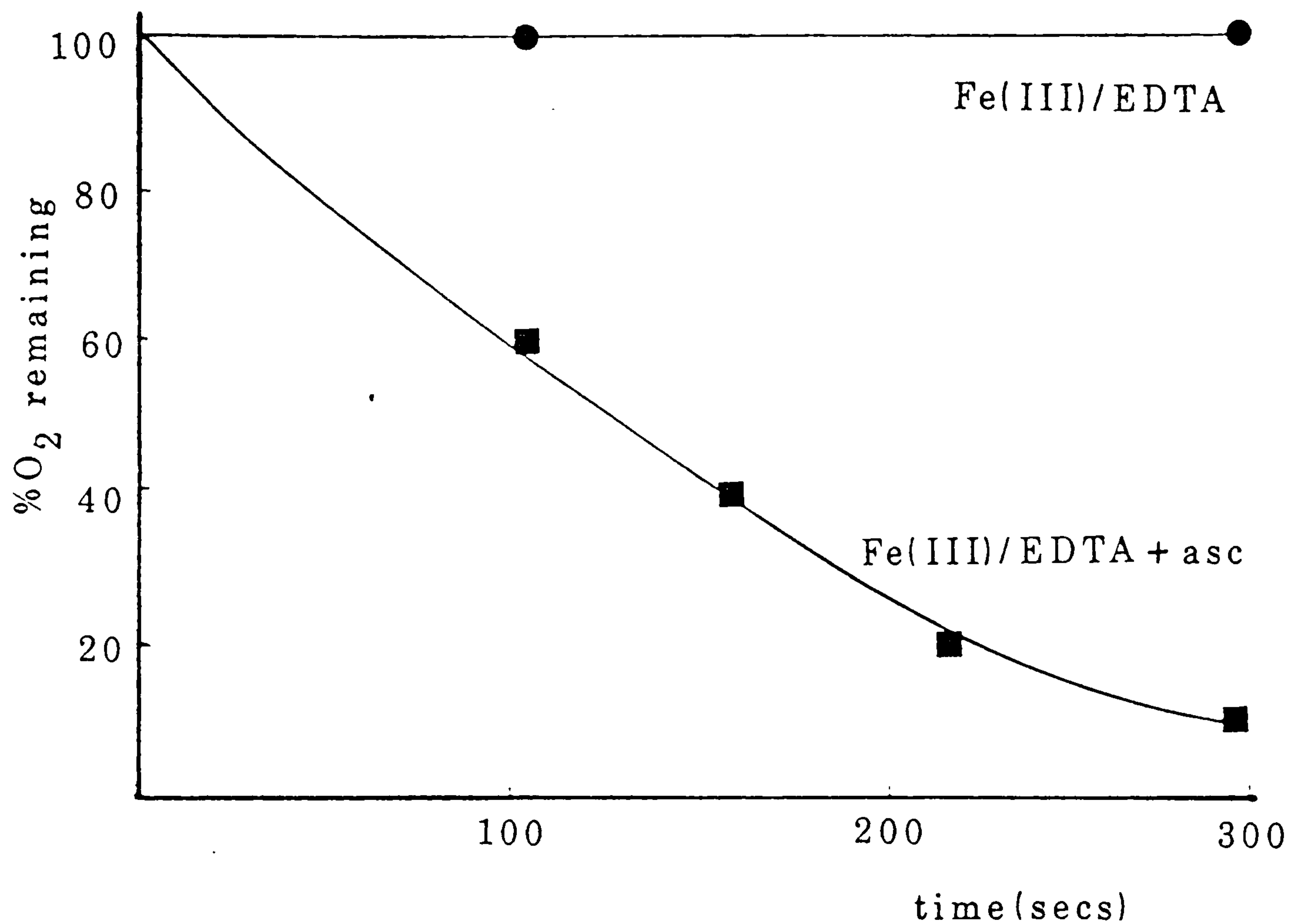


fig 2.12

RATE OF O_2 CONSUMPTION IN THE PRESENCE OF $FeCl_3$/EDTA +/- ASCORBATE



$FeCl_3$/EDTA 1:1 100µM

ascorbate 5mM

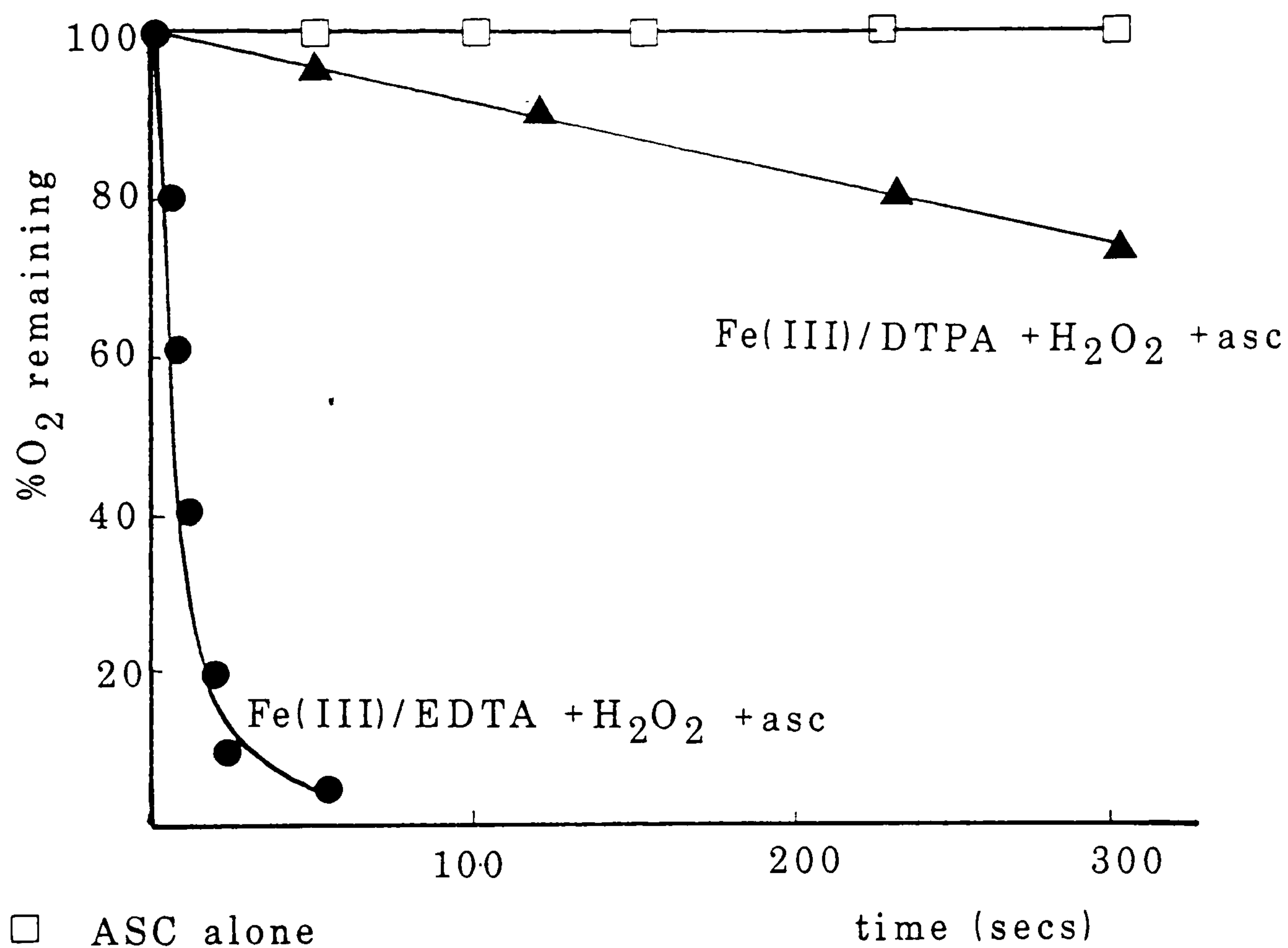
inPBSi pH 7.40

fig 2:13

O_2 consumed using an oxygen electrode. H_2O_2, (5 mM) was added directly after addtion of 100μM Fe/complex (1:1) in a solution of PBSi, pH 7.40, followed by addition of 5 mM ascorbate. In the case of Fe(III)/dex the final iron concentration was 9 mM. This had the effect of increasing the rate of oxygen utilisation. The values were significantly greater than the rate in the presence of any of the agents alone. The rate of oxygen utilisation under these conditions varied according to the nature of the complexing agent. Fig 2.14 shows the rate of oxygen utilisation of 3 different iron complexes and illustrates that the ligand has a significant effect on the half life of O_2 under these conditions. For example, DFO prevents O_2 consumption, whilst the half life of O_2 when iron is complexed to DTPA is 50 times longer (9.33 mins) than when the ligand is EDTA (10 secs). To some extent this is influenced by the rate of reduction by ascorbate since, when the ligand complexes are made with Fe(II) the rates of O_2 utilisation are increased (fig 2.15). Decreasing the concentration of $FeCl_3$/EDTA reduces the rate of oxygen utilisation (fig 2.16) and no O_2 is used in the absence of iron over the time scale measured.

Fe/dextrans also utilise O_2 under these conditions. Fig 2.17 shows a comparison between the oxygen utilisation of freshly opened Fe/dex (16H) with Fe/dex (8K) previously exposed to air for more than one week. The half life of O_2 for batches 16H and 8K were 7 secs

RATE OF O_2 UTILISATION BY $FeCl_3$ COMPLEXES INTHE PRESENCE OF H_2O_2 AND ASCORBATE



□ ASC alone

and similar data for:

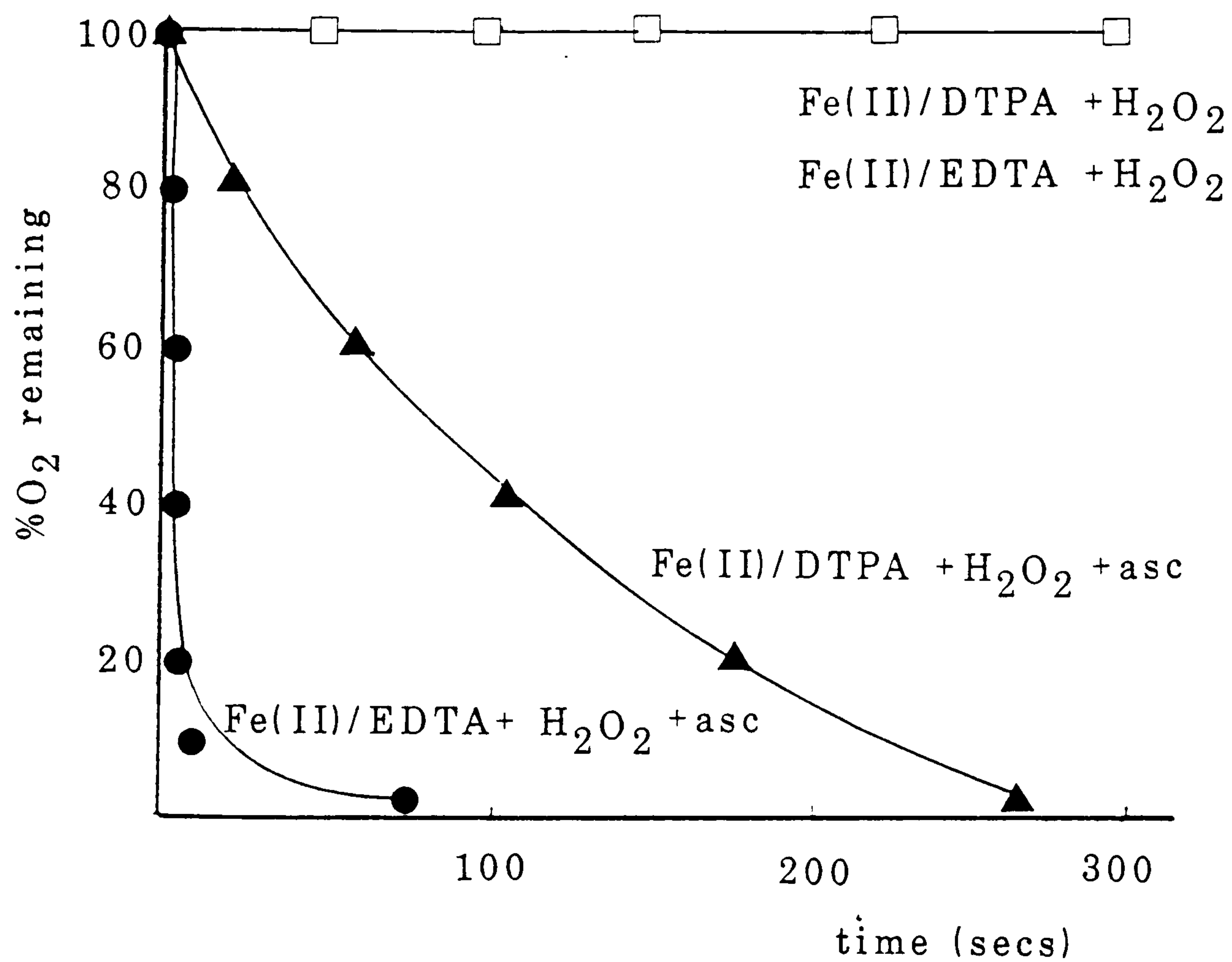
- H_2O_2 + ASC
- Fe(III)/EDTA + H_2O_2
- Fe(III)/DTPA + H_2O_2
- Fe(III)/DFO + H_2O_2
- Fe(III)/DFO + H_2O_2 + ASC

Fe(III)/C (1:1)	100µM
H_2O_2	5mM
ASC	5mM

PBSi pH 7.40

fig 2.14

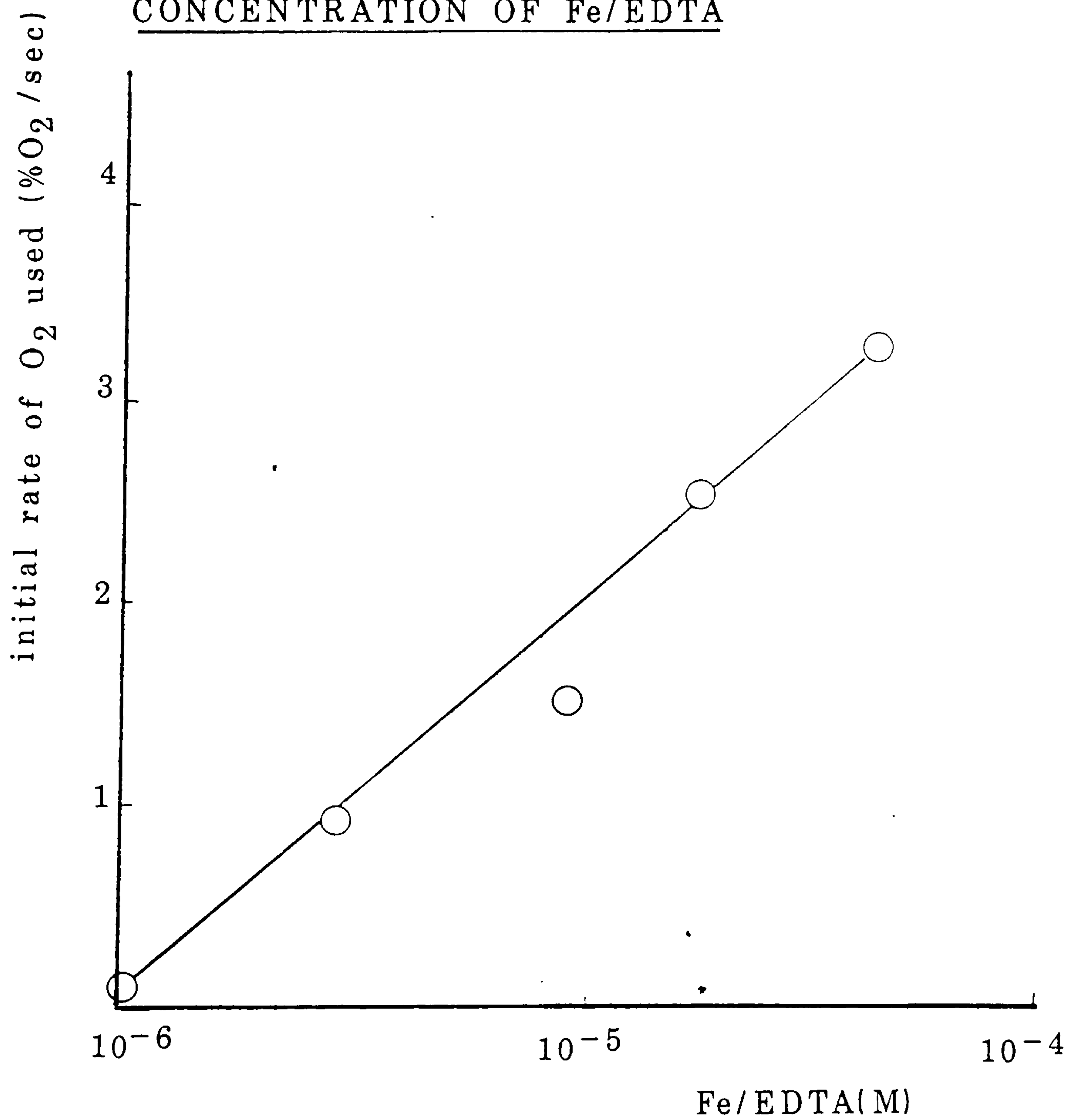
RATE OF O_2 UTILISATION BY $FeSO_4$ COMPLEXES IN THE PRESENCE OF H_2O_2 AND ASCORBATE



Fe(II)/C (1:1)	100µM
H_2O_2	5mM
ASC	5mM

PBSi pH 7.40

fig 2.15



H_2O_2 5mM

ASC 5mM

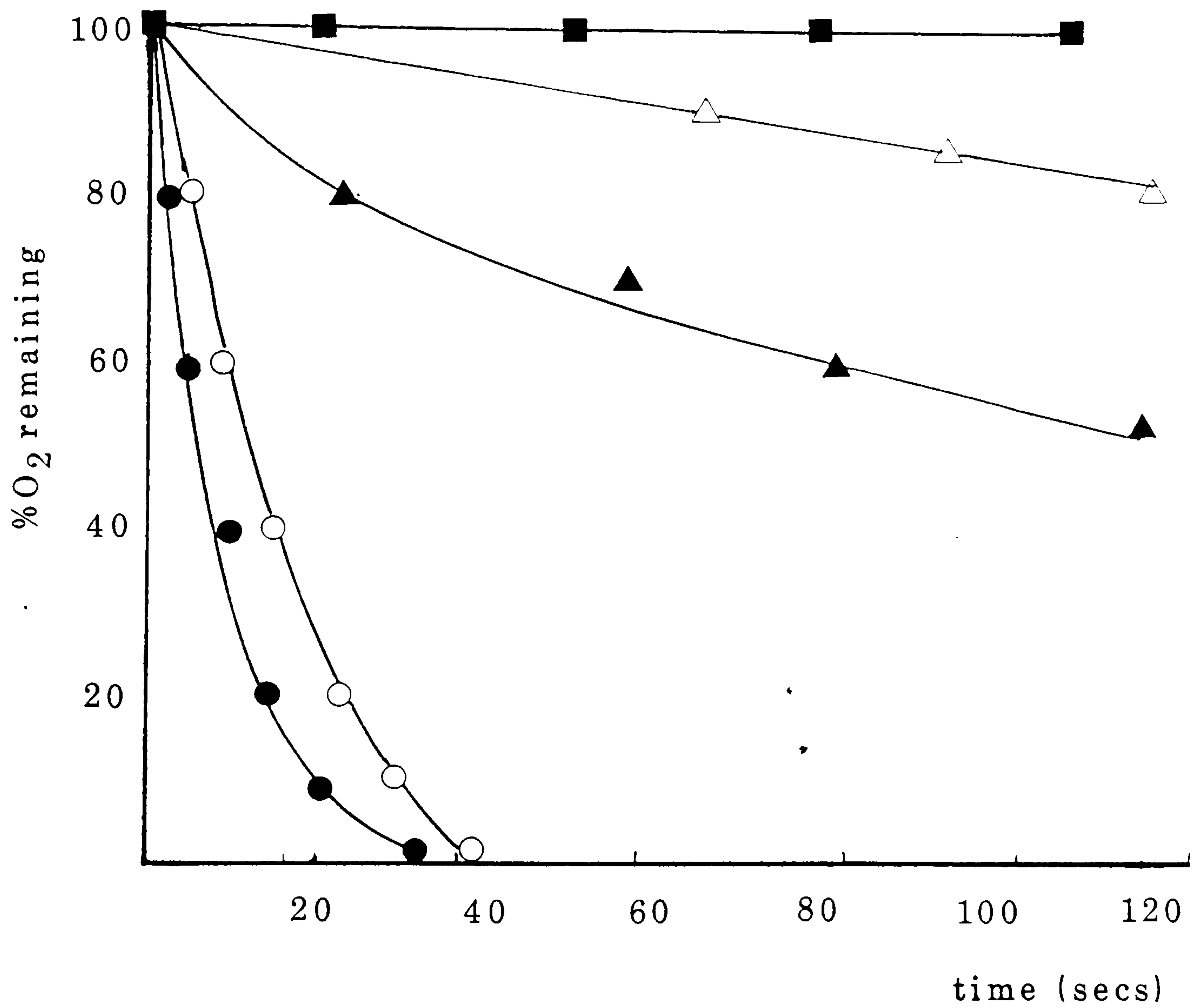
PBSi pH 7.40

fig 2.16

Legend to fig 2.17:

batch 16H freshly opened
batch 8K opened 1 month prior to measurement
H_2O_2 5mM
ASC 5mM
Fe/dextrans 9mM (Fe)
PBSi pH 7.40

RATE OF O_2 UTILISATION IN THE PRESENCE OF H_2O_2, ASCORBATE AND Fe/DEX



■ batches +/− H_2O_2

△ 8K + ASC

▲ 16H + ASC

○ 8K + H_2O_2 + ASC

● 16H + H_2O_2 + ASC

fig 2.17

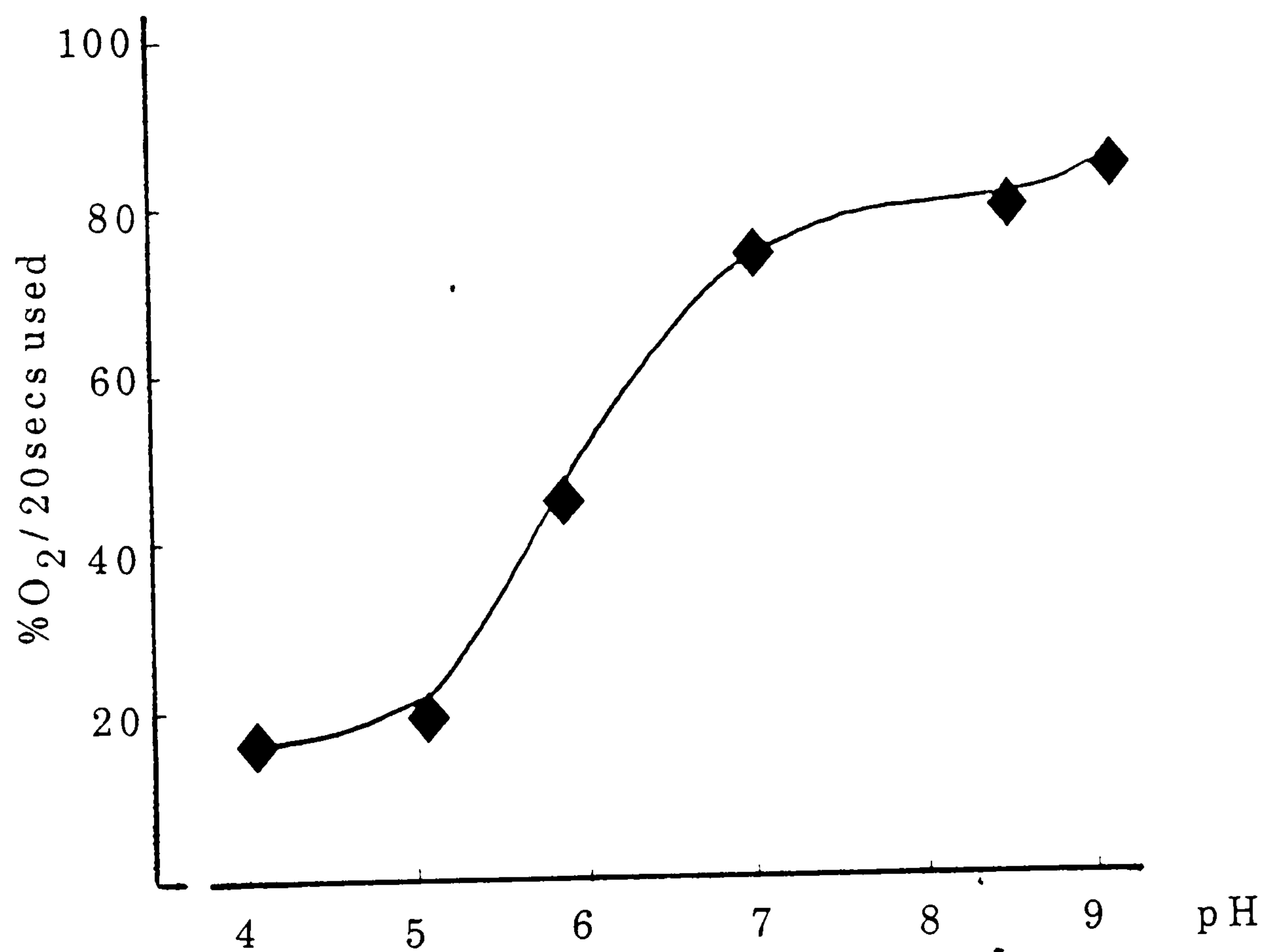
and 11 secs respectively in the presence of H_2O_2 and ascorbate. The pH dependence of oxygen utilisation by Fe/dex is illustrated in fig 2.18 and is most rapid in neutral to alkaline conditions.

In the presence of H_2O_2 reducing the concentration of ascorbate did not affect the initial rate of utilization but altered the amount of O_2 used. By contrast, in the presence of excess ascorbate, only the rate of O_2 consumption by Fe/dex changes when the concentration of H_2O_2 is lowered, and the O_2 utilisation goes to completion, fig 2.19.

2.3.2.9 H_2O_2 concentration

The concentration of H_2O_2 was measured as described in section 2.2.6.1 under 4 different conditions as shown in table 2.7. The results show that in the presence of $FeCl_3$/EDTA the H_2O_2 concentration was unchanged. Since 48µM of H_2O_2 reacted with 50µM ascorbate, there is apparently a 1:1 stoichiometry between H_2O_2 and ascorbate. The total amount of O_2 used as a function of ascorbate concentration under these conditions is shown in fig 2.20. This indicates that Fe/EDTA is acting as a catalyst for the reaction between H_2O_2 and ascorbate.
The effect of pH on the same system of 5mM H_2O_2, 5mM ascorbate and 100µM $FeCl_3$/EDTA is similar to that observed for Fe/dextrans, (fig 2.21) where O_2 utilization increases at higher pH.

pH PROFILE OF Fe/DEX IN THE PRESENCE OF H_2O_2 AND ASCORBATE



Fe/dex batch 8K 9mM

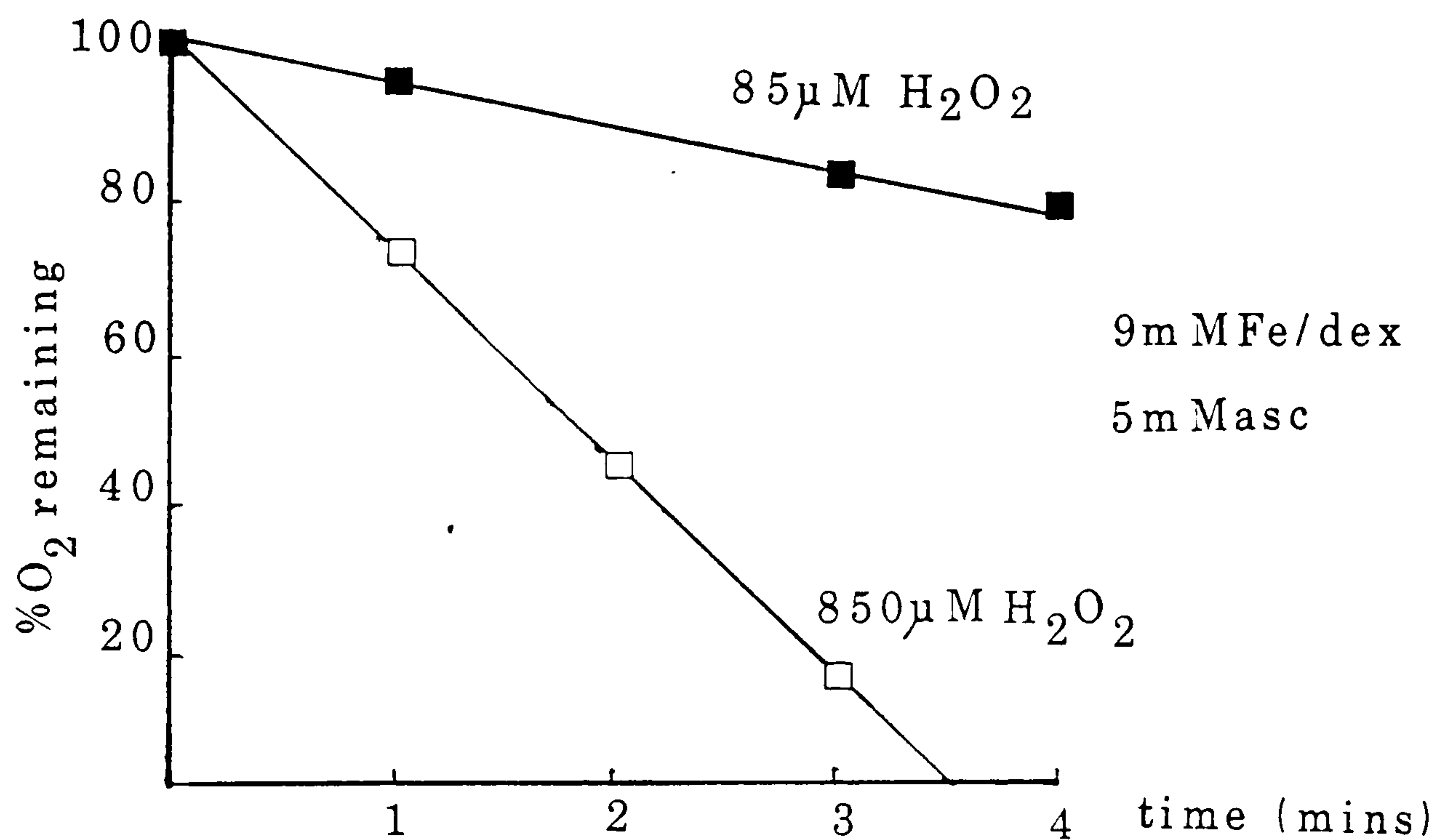
H_2O_2 5mM

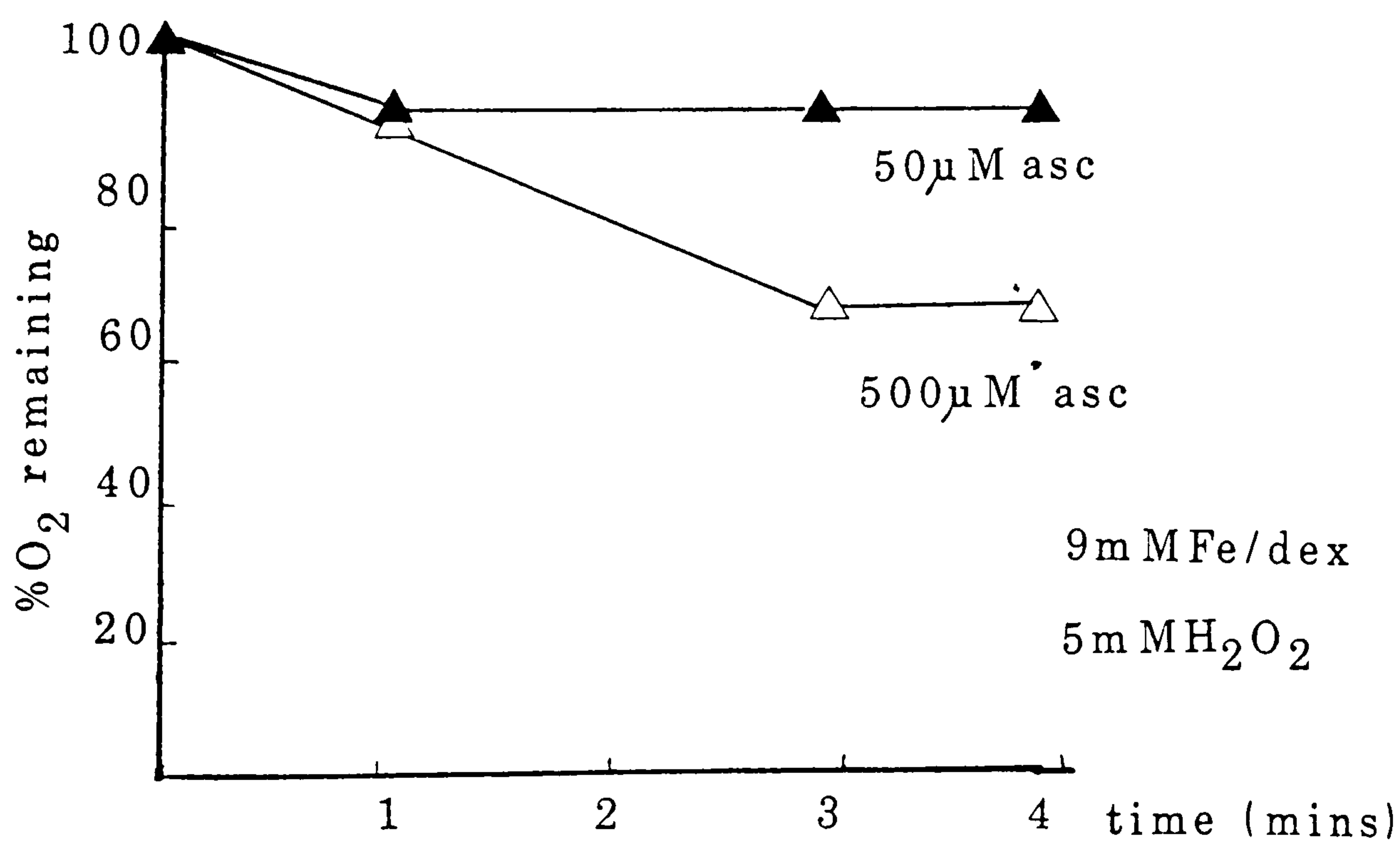
ASC 5mM

PBSi

fig 2.18

EFFECT OF CHANGING THE CONCENTRATION OF H_2O_2 OR ASCORBATE ON THE RATE OF O_2 UTILISATION





PBSi pH7.40

fig 2.19

TABLE 2.7

H_2O_2 CONCENTRATION REMAINING UPON ADDITION OF Fe/EDTA AND/OR ASCORBATE

additions[1]	H_2O_2 (μM) remaining
-	74
Fe(III)/EDTA[2]	74
ascorbate[3]	26
Fe(III)/EDTA + asc[4]	26

[1] All solutions contained H_2O_2 at the same initial concentration

[2] To this solution of H_2O_2 100μM Fe(III)/EDTA (1:1) was added and the H_2O_2 concentration measured by the KI method.

[3] To a solution of H_2O_2 50μM ascorbate was added and the H_2O_2 concentration measured.

[4] The final solution contained 3 components: H_2O_2, Fe(III)/EDTA and ascorbate and the remaining concentraion of H_2O_2 measured.

EFFECT OF ASCORBATE CONCENTRATION ON THE O_2 UTILISATION

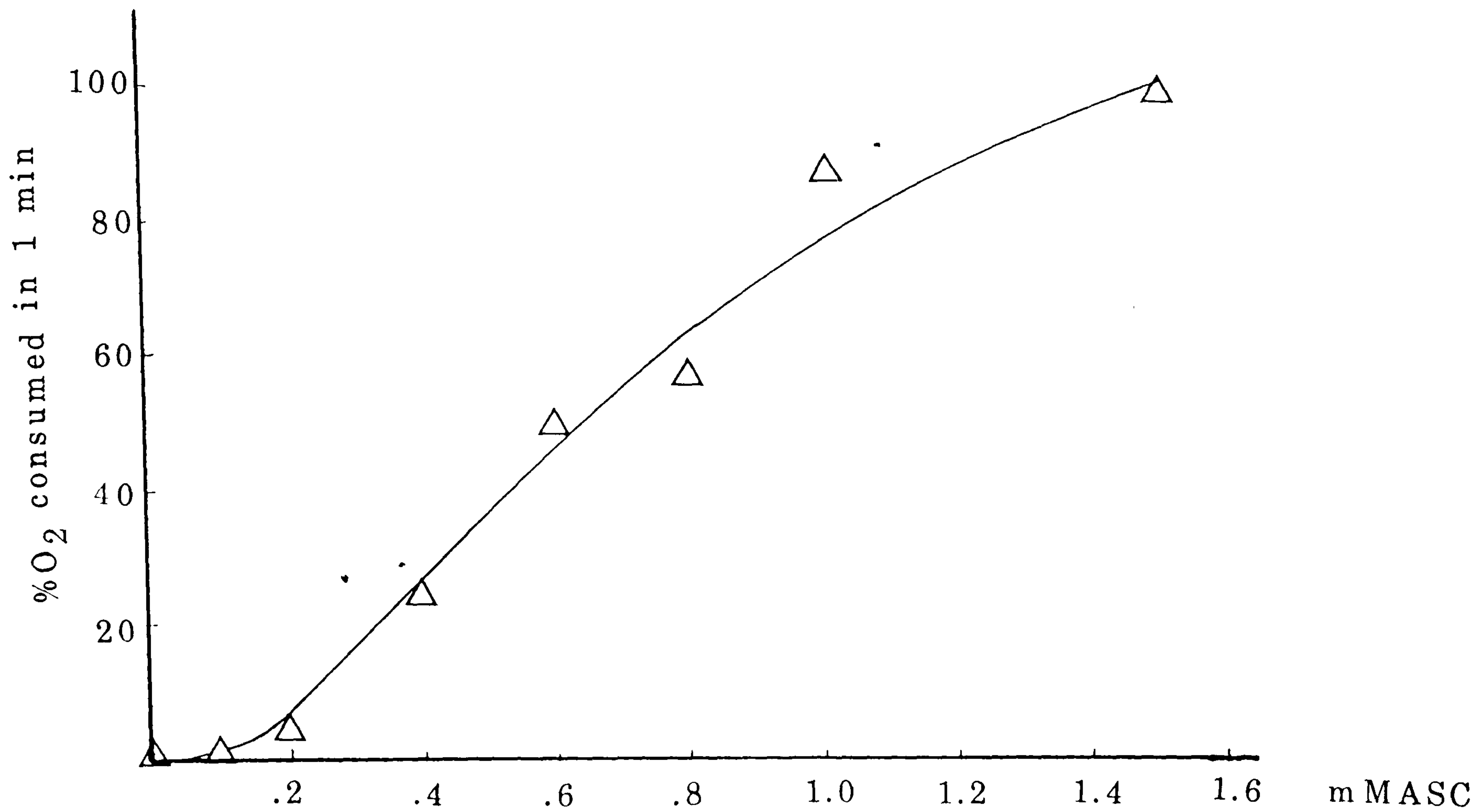


fig 2.20

Fe(III)/EDTA 100µM ; H_2O_2 5mM ; PBSi pH 7.40

EFFECT OF pH ON THE HALF LIFE OF O_2 IN THE ASCORBATE/H_2O_2/$FeCl_3$/EDTA SYSTEM

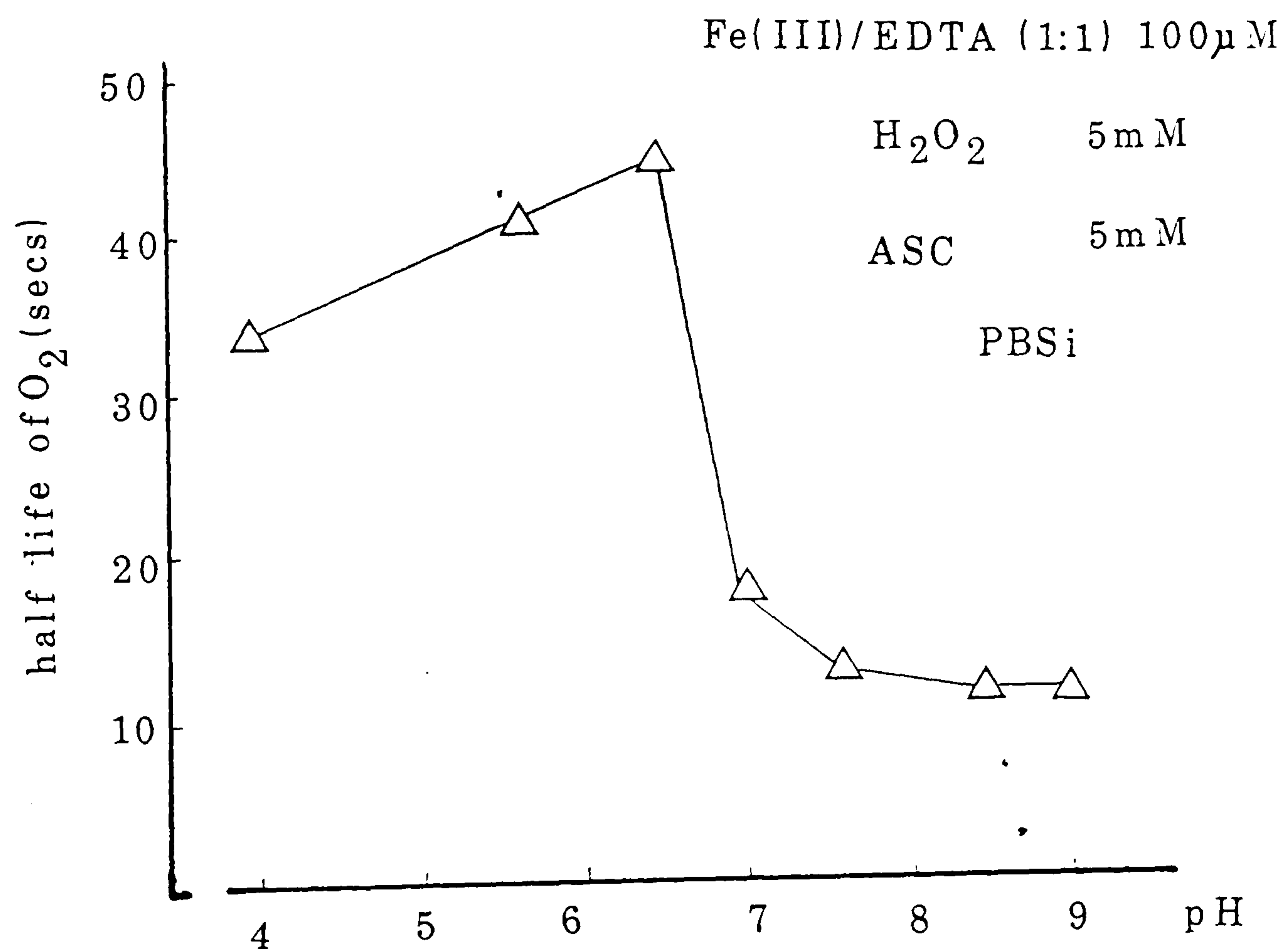


fig 2.21

2.3.2.10 Half-life of H_2O_2 in the presence of iron complexes

The rate of reaction of H_2O_2 with Fe^{2+} was influenced by the nature of the complexing agent.

This could be tested by measuring the amount of H_2O_2 remaining in a solution containing PBSi, Fe(II)/ATP or Fe(II)/EDTA and H_2O_2 using the KI method. The initial concentration of Fe^{2+} was 100 µM and the iron to ligand ratio was 1:5. Solutions were made up in test-tubes, placed into a 37°C incubator and aliquots removed at time intervals between 0 and 60 minutes. It was not possible to measure the half life of H_2O_2 in the presence of certain coloured complexes using the KI assay as these were found to interfere with the absorbance measurements.

The half lives were very short: 1.2 minutes for Fe^{2+}/EDTA and 0.3 minutes for Fe^{2+}/ATP (fig 2.22). After ca. 5 minutes the rate of removal of H_2O_2 reaches a plateau. This may be due to the limited availability of the Fe(II) complex since, as is demonstrated in section 2.3.2.1, the rate of autoxidation of these complexes was rapid.

Legend to fig 2.22:

Freshly prepared Fe complexes: 100μM Fe; 500μM ligand was immediately added to 50μM H_2O_2 in PBSi pH 7.40 at 37°C.

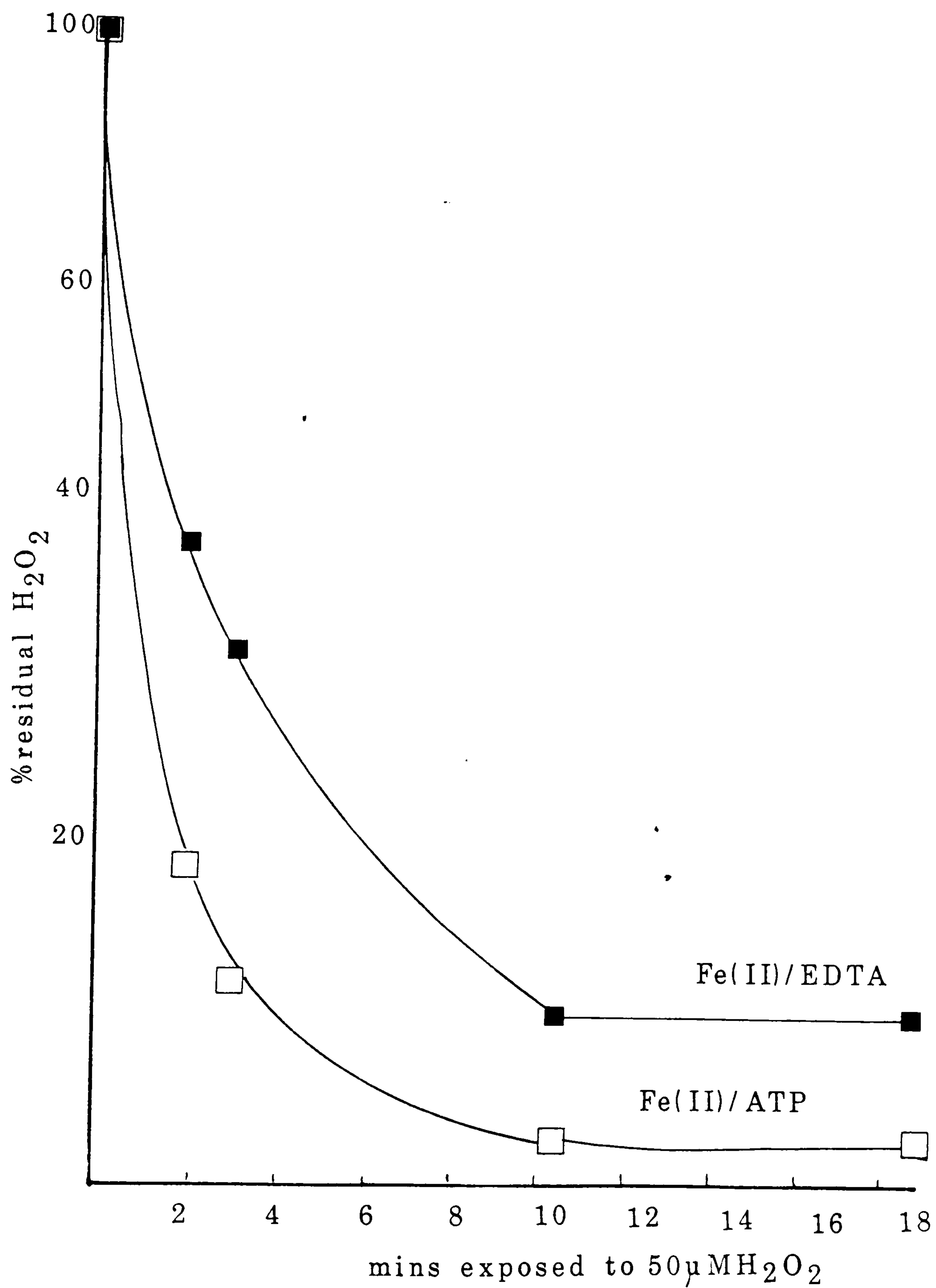


fig 2.22

2.4. DISCUSSION

Since it was anticipated that the cytotoxic action of iron would be influenced by the redox state of the iron it was considered important to investigate the relative stability of Fe-ligand complexes. Both O_2 utilisation and measurement of residual Fe(II) by ferrozine showed that in most complexes the metal tended to the oxidised state. The rate of oxidation was found to be fairly rapid; ($t½$ of O_2 utilisation was respectively: PCA=10s; DFO=2.5s; EDTA=13s; DTPA=17min; ATP=17min; 1-His=4min; imidazole=4min; AMP=1.8min; ADP=1.1min). DTPA and ATP were surprisingly slow, but this may be due to a combined effect of the imidazole buffer and the ligand. As expected, both phenanthroline and ferrozine inhibited oxidation of Fe(II) over the time scale measured.

The preferred oxidation state of iron in various ligand complexes is determined by the nature of the ligand-metal interactions. The electron affinity (expressed as the redox potential) of aqueous oxygen is +1.23 eV (from the Nernst equation). The redox potential of the reaction Fe(II) $\longrightarrow$ Fe(III) + e^- is -0.77 (where the ligand is water). Thus, the net redox potential for aqueous Fe(II) in the presence of oxygen is +0.46 and the iron will tend to be oxidised. Electrons donated in coordination bonds tend to increase the electron density of the metal and hence increase the net positive redox potential of the Fe(II)/O_2 coupled reaction. For example,

the redox potential of Fe complexed to $(CN)_6$ is -0.36 compared to -0.77 for $(H_2O)_6$. This effect of ligands is more marked for spin paired electrons, as in the case of coordination bonds with nitrogen (↑↓), than for unpaired electrons, as in the case of oxygen (↑↑), because any electrons which are donated must also be of parallel spin to fit into the vacant spaces in the Π* orbitals (Nyholm & Tobe,1963). Hence, the accelerated O_2 uptake by Fe(II)/EDTA etc. is the consequence of ligand affecting the redox potential of iron.

When the ligand has a structure that places suitable Π orbitals in the range of the d orbitals of the metal, delocalisation of metal electrons (so-called "back-bonding", Albert, 1986) may be favoured with concomitant stabilisation of the coordination complex and diminuition of the net electron density of the metal ion. This electron (dense) 'cloud' is shared between the metal and the ligand. This is the case, for example, with Fe(II) complexed to phenanthroline, in which back-bonding occurs. The resultant redox potential of iron is -1.12 (ie a net redox potential of +0.11 in the presence of oxygen).

Whether or not a ligand can facilitate reactions with iron depends additionally on factors such as the binding constant and the number of coordination sites the ligand occupies. For instance it has been suggested that the inability of DTPA or DFO to facilitate the

superoxide-driven Fenton reaction (Halliwell,1978; Gutteridge et al.,1979; Beuttner et al.,1983) may be due to all the coordination sites being occupied (Graf,1984). The reactivity of an iron complex with a very high binding constant will also be reduced due to sharing of the electron density between the metal and the ligand (see table 2.8 for stability constants and redox potentials).

In anticipation of a suitable buffering medium it was decided to exploit the properties of an imidazole buffer which has been suggested to retain the Fe(II) form of iron better than other types of commonly used buffers (Lambeth et al., 1982). This may be due to a possible ligand effect induced by the imidazole ring (Paques et al.,1980). In this context studies with nitro compounds such as misonidazole (Samuni et al., 1986) have suggested that their biological effects may be subject to modification by modulators of iron metabolism, eg. thiols or reduced co-enzymes. These studies have shown that the effect of these compounds on cells are modified by the addition of iron complexes. Also, metronidazole toxicity to trichonomas vaginalis is enhanced by the addition of ferrous iron in vitro (Meingassner & Heyworth, 1982). Imidazole-buffer was not used frequently for cell incubations in the following chapters, because this buffer caused some impairement to cell viability.

It was demonstrated that O_2 utilisation by

TABLE 2.8

EXAMPLES OF STABILITY CONSTANTS OF SOME IRON COMPLEXES

Ligand	*K Fe^{3+}	K Fe^{2+}	E_o
H_2O	?	?	- 0.77
EDTA	$^{1}24$	$^{1}14$	- 0.14
8-HQ	$^{1}12, ^{2}24, ^{3}36$	$^{8}, ^{2}15$	$-^{1}0.52$, $-^{2}0.27$
o-PHEN	$^{3}14$	$^{3}21$	$-^{3}1.06$
BIPYRIDYL	?	$^{1}4.5$	$-^{3}1.06$

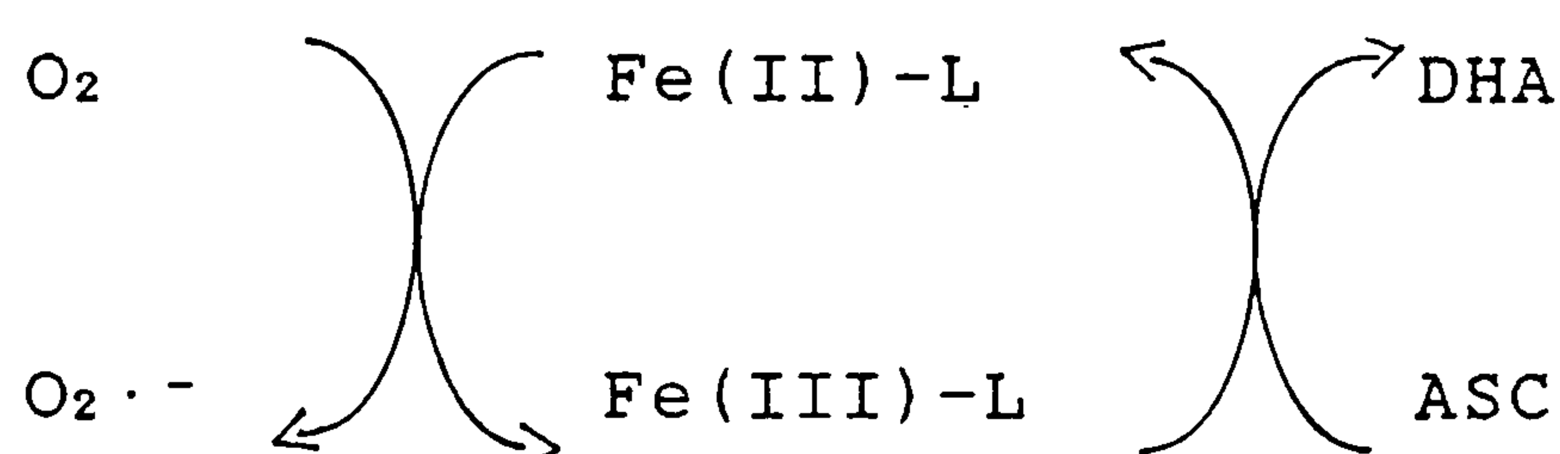
*K = binding, or stability constant (it is essentially an equilibrium constant)

$^{1}, ^{2}, ^{3}$ prefixes represent the number of ligand per ionic iron.

E_o = potenial in volts (at 20°C)

references: (Irving & Mellor,1962; Sillen & Martell,1964; Perrin,1959; Hawkins & Perrin,1962)

Fe(II)/complex is stimulated by the addition of ascorbate and that this stimulation is dependent on the total amount of ascorbate added. The results are consistent with a metal-ligand catalysed oxidation of ascorbate to DHA:



This was shown effectively by both by Fe/dex and Fe/EDTA. In this reaction O_2 uptake was stimulated by the addition of H_2O_2. A mixture of H_2O_2 and ascorbate resulted in a 1:1 stoichiometry and was unchanged by the addition of Fe/EDTA. However, the rate of O_2 utilisation in the presence of Fe/EDTA or Fe/dex was considerably faster. This also increased with increasing H_2O_2 concentration. Since H_2O_2 is a strong oxidising agent which reacts rapidly with Fe(II) by the Fenton reaction (McCord & Day, 1978; Halliwell,1978) this is surprising. The catalytic activity of Fe/EDTA in the H_2O_2/ascorbate couple system has been demonstrated previously by Undienfriend et al. (1954) in which the hydroxylation of aromatic compounds was stimulated.

A possible explanation for this catalysis might be that the ligand is altered by hydroxyl radicals generated from H_2O_2 yielding a modified Fe/complex with a lower

redox potential (ie a greater net positive potential in the presence of O_2). Ligand attack by hydroxyl radicals is a subject of current investigation (Goldstein, 1988). In the case of Fe/dex ligand modification is implied by heating with ascorbate (see fig 2.12). Indeed, recent evidence from ESR experiments employing a flow system in conjunction with the Ti(III)-H_2O_2 couple shows that dextrans react with the HO· radical via indiscriminate attack (Gilbert et al.,1984).

CHAPTER III: LOCALISATION OF IRON IN CELLS AND THE EFFECT ON CELL SURVIVAL

3.1 INTODUCTION

Taking into account the physico-chemical properties of iron complexes studied in chapter II, this chapter examines the effect of some of these complexes on cells in vitro. Both the redox state of iron and the lipid permeability which contribute to the distribution of iron within the cell may be factors which influence the cellular response towards different iron complexes. Depending on the localisation of cell-associated iron consideration is given to any cytotoxic effects the iron complexes may exert.

3.2 MATERIALS AND METHODS

All iron complexes used for cells were prepared as described in section 2.2 but all solutions were filter-sterilised prior to addition to cells. Phosphate buffered saline (PBSi) was prepared for incubation as described in section 2.2. PBS A+B (Oxoid Ltd.) was used for washing the cells and was sterilised by autoclaving.

The following section deals with the general methods for tissue culture which also applies to chapter IV.

3.2.1 General cleaning procedure

All glassware and plastics (Falcon flasks, Petri dishes, bijoux and universal bottles) used for tissue

culture were washed as follows: immersion in cleaning liquid (Decon) overnight (o/n), rinsed with tap water, left o/n in 2% HCl, washed thoroughly with tap water, rinsed 3 x in distilled water and dried.

Graduated pipettes were placed in 2% bleach (Chloros) o/n, rinsed in tap water, rinsed with distilled water and dried. Caps were rinsed, placed in distilled water containing detergent (Fairy liquid) and boiled for 5 minutes, rinsed in distilled water and dried.

The work area was swabbed down with Precept (which contains 1000 ppm available chlorine) and a solution of Fairy liquid. All liquid waste was emptied into 75% Chloros. Precept and Chloros were changed weekly. 1% Hycolin was used for general cleaning of the sterile laboratory.

3.2.2 Sterilising

Non-plastic or non-rubber was sterilised by hot air oven at 160°C for 2 hours. Plastic caps, pipette disposable tips, plastic tubing etc. were autoclaved for 15 minutes. Disposable plastics, ie. falcons etc. were sterilised by gamma irradiation. Pipettes were plugged with non-absorbant cotton wool, placed in canisters and sterilised by hot air oven.

3.2.3. Culture medium

The culture medium used was minimum essential medium,' Eagle (modified) with Earle's salts (Flow Labs). This medium was supplemented with 20 mM N-2-

hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes buffer, Flow Labs), 10% foetal bovine serum (Imperial Labs), 0.4 mM L-glutamine, penicillin (10u/ml) and streptomycin (10μg/ml).

3.2.4 Cell Line

A line of mammalian epithelial cells, of human breast origin, accession no. CNCM I.221, (Collection Nationale de Cultures de Microorganisms - Institute Pasteur, Paris) established in 22.3.1972, was used.

The starting passage number used was 21. The cells were removed from storage in liquid nitrogen, reconstituted by rapid thawing under warm water and placed in growth medium. The medium was changed the following day. After the first subculture aliquots of cells were removed for (a) further subculture,
(b) preparation of an autoradiograph to test for mycoplasma; and (c) storage by freezing.

3.2.5 Freezing

Cells were protected from low temperature damage by the addition of dimethylsulphoxide (DMSO) to the medium. The cells were spun down and the pellet resuspended in 7.5% DMSO to give a cell concentration of about 2 x 10^6 cells/ml. The suspensions were transferred in 1-2 ml aliquots to sterile 2 ml polypropylene tubes (cryogenic ampoules, R & L Slaughter Ltd) and placed in a polystyrene container. Stepwise freezing was carried out for 3 hours or overnight at 4°C, followed by 30 minutes

at -18°C, overnight at -70°C and the ampoules finally transferred to liquid nitrogen for long term storage.

3.2.6 Autoradiography

Autoradiography was carried out routinely whenever cells were removed from liquid nitrogen. Under sterile conditions cells were seeded at 5 x 10^4 cells/ml onto a glass microscope slide and incubated in a CO_2 incubator at 37°C for 2-3 days. 50µl of ^{3}H-TdR (^{3}H-[methyl]-thymidine, specific activity 5 mCi/mmol, Amersham International Plc) was added for 5 hours at a final concentration of 5 µCi/ml in growth medium. The cultures were then washed with PBS and fixed in 4% gluteraldehyde for 10 minutes. Gluteraldehyde was prepared by diluting 4 ml of a 25% stock solution (TAAB Laboratory Equipment Ltd.) into 25 ml Sorensen's buffer pH 7.4 (19.6 ml 6.7 x 10^{-2} KH_2PO_4, 80.4 ml 6.7 x 10^{-4}M Na_2HPO_4). The specimen was then washed with distilled water and dehydrated through, 70%, 90%, 95% and 100% ethanol. Passage number, date and cell line were marked on the underside and the slide was then coated with photographic emulsion, exposed for 1 week and developed in the darkroom.

3.2.6.1 Coating

A heaped spatula of photographic emulsion (Emulsion K2 in Gel form, Ilford Ltd.) was mixed with an equal volume of distilled water (previously heated to 37°C in a water bath) to form a thin suspension and kept warm. The emulsion was poured the upper surface of the slide which

was angled to allow the emulsion to run down it to form an even coat. The slide was left to dry for 10 minutes and then transferred to a light-tight box, sealed with tape and left at 4°C for one week.

3.2.6.2 Developing

Developer (Contrast FF, Ilford Ltd.) was diluted by addition of 15 mls to 100 mls distilled water and poured into the slide box and mixed every 5 minutes for 15 minutes. The slide was then washed with distilled water and ammonium thiosulphate (Hypam fixer, Ilford Ltd.) added and left for. 5 minutes with mixing. The slide was then thoroughly washed with tap water.

3.2.6.3 Staining

Staining was carried out for 20 minutes using freshly filtered Giemsa R66 (BDH chemicals). The slide was washed in distilled water and dehydrated through 70%, 95%, and absolute alcohol. Xylene was used as a clearing agent and the specimen mounted in Depex: The specimen was inspected for evidence of mycoplasma contamination which is characterised by diffuse surface labelling of the cells and inhibition of nuclear labelling. Cultures in which no evidence of mycoplasma contamination was observed were used for experiments. All the cultures employed for the work reported in this thesis were free of contamination.

3.2.7 Trypsin

Subculturing was carried out routinely every week

using 5% trypsin (Trypsin powder: Hopkin & Williams 883600) from a 10% stock solution (in 6mM glucose and 1mM phosphate buffer) which was diluted with serum free medium.

3.2.8 Versene

EDTA 0.02% (Versene, Flow Laboratories) was also used initially to avoid possible interactions with membrane proteins which may influence the results. However, it did cause considerable 'clumping' of cells and was only used in the early studies to prevent any introduction of inaccuracy in the counting of cells.

3.2.9 Details of subculture

Before subculturing the cells were inspected for general appearance and morphology using an inverted phase-contrast microscope (Nikon).

The medium was poured off and replaced with 5% trypsin solution and placed in a CO_2 incubator at 37°C and inspected after a few minutes. When the cells had detached they were removed using a sterile pipette and placed into a glass centrifuge tube and any residual cells were washed into the tube with growth medium. The cells were spun down using a bench centrifuge at approximately 800 rpm (g=45.5) for 3 minutes. The supernatant was decanted off and the cells resuspended in 1 ml of medium using a 1 ml syringe to disperse them. 20µl of the cell suspension was removed placed into 10 mls of saline and counted using a Coulter counter (model

ZB1). Appropriate dilutions of the remaining suspension were made to give a final concentration of 10^6 cells/ml. This was used to prepare cultures of cells at different cell densities.

3.2.10 Doubling Time

From previous experience with this cell line the average doubling time was known to be between 16 and 24 hours for lower passage numbers but was found to increase with increasing passage number. It was found that a seeding density of 1-2 x 10^3 cells/ml at the beginning of the week gave rise to a confluent monolayer after 8 days in either a small falcon of 25cm^2 growth area (with a total of 2-3 x 10^6 cells) or a large falcon of 75cm^2 growth area (with 9-12 x 10^6 cells). On average the cells have divided 8-9 times in 8 days when seeded at 10^3 cells/ml.

Passage numbers were allowed to reach a maximum of 50. Thereafter cells were reconstituted again from the lower passage number.

3.2.11 Plating Effiency: colony formation,(relative)

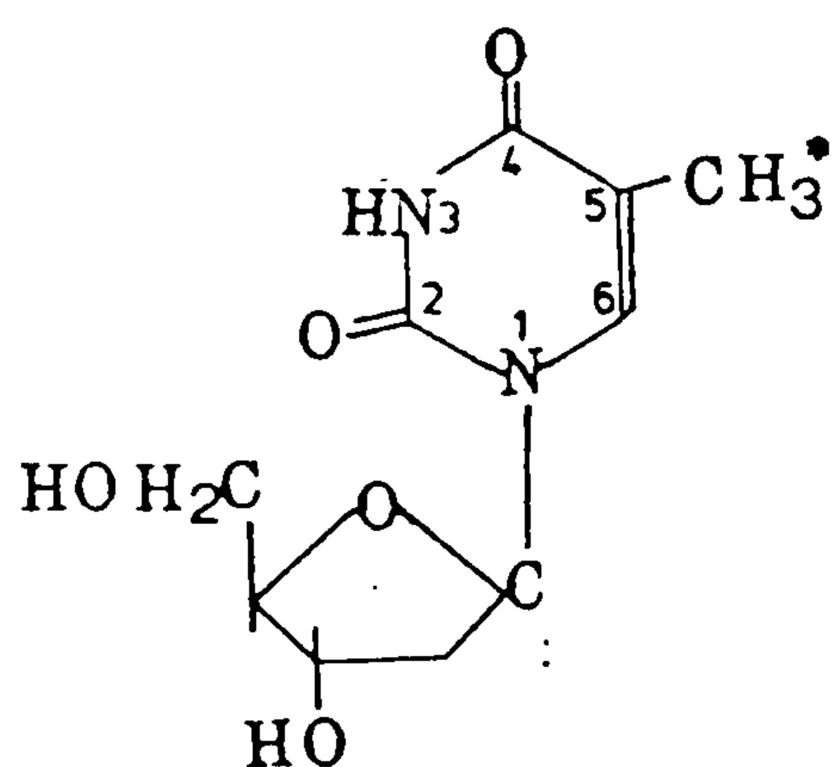
Each experiment was carried out with cells exposed to the agent in suspension. Cells from a stock falcon (Falcon plastics, Scientific Supplies) were removed by versenation and appropriate dilutions made to give between 5 x 10^4 cells/ml and 2 x 10^5 cells/ml depending on the hature of the experiment. For each experiment 1.0 ml of cell suspension was placed into 2 ml polypropylene

ampoules and incubated at 37°C on a continuous rolling mixer (Denley Spiramix) for 60 minutes to ensure homogeneous mixing. After the experiment the cells were counted using a haemocytometer (Neubauer grating type). At this stage the general appearance and morphology of the cells were noted if affected by the agent. The number of cells in each chamber (9 in total) were multiplied by 10^4 and the mean of 9 chambers taken as the final count. From these counts serial dilutions were made with growth medium, the final dilution of which was calculated to give 200 cells per petri dish (Nunc 5cm diameter, Inter Med) containing 5 mls of medium. This last step was carried out in triplicate. Light shaking ensured even distribution. The petri dishes were then placed into a pre-warmed moist plastic box and left in a CO_2 incubator at 37°C for 8 days, by which time large enough colonies had formed to enable them to be counted. The medium was carefully removed, the dishes washed twice with PBS A+B and the cells fixed with absolute alcohol for 5 minutes. This was replaced by absolute alcohol containing crystal violet (George T.Gurr Ltd) (approx. 2g/500ml absolute alcohol) usually overnight. Colonies were counted using a low power binocular microscope. Colonies were counted if they contained more than approximately 50 cells. The mean of triplicate estimations was used to calculate the survival, expressed as the percentage of the number of colonies formed compared to untreated controls.

3.2.12 Thymidine Incorporation Assay

3.2.12.1 Backround

The method involves the measurement of the incorporation of radiolabelled thymidine into DNA. Thymidine enters the cell and is phosphorylated to thymidine monophosphate. After conversion to thymidine triphosphate it is incorporated into DNA. Under most circumstances thymdine incorporation is regarded as a measure of DNA synthesis. The standard labelling procedure uses TdR with the ^3H atom being located in the methyl group (Pastore & Friedken, 1962).



(I)

Measurement of thymidine incorporation depends on DNA extraction which is usually based on dissolving the 5% TCA-insoluble material from cells in sodium hydroxide (1M). The radioactivity of this material is estimated by scintillation counting, which depends on the generation of photons by collision of the electrons emitted by the tritium decay with suitable light emitting molecules.

3.2.12.2 Detection of Radiolabel by Scintillation Counter

The light output of a scintillation mixture depends upon the conversion of energy by a series of energy

transfer steps starting from a β-particle emitted by the radioisotope (^{3}H). This reacts with a series of organic solvents which trap the energy and convert it to photons at a wavelength which can be detected by the 2 photomultiplier tubes. The steps involved in the scintillation fluid used for the experiments reported are shown in scheme 1.

3.2.12.3 Labelling of Cells

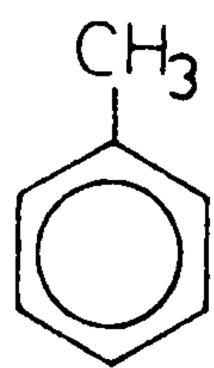
A stock solution of tritiated thymidine (^{3}H-TdR, [methyl-^{3}H]-Thymidine, specific activity of 5 mCi per mmol) was obtained from Amersham International Plc. This was diluted with sterile PBS A+B to give a stock solution of 20µCi/ml. Cells were seeded at different densities with 1 ml of growth medium in multiwell trays, each having a diameter of 1.5 cm (1.77cm^{2}) (Falcon Plastics, Scientific Equipment), and exposed to the agents the following day.

Thymidine incorporation was carried out by addition of 1 µCi/ml final concentration of ^{3}H-TdR (ie. 100µl stock solution per 1 ml medium in each well) and incubation for 30 minutes at 37°C. The cells were then washed 5 times in non-sterile PBS, fixed with 5% TCA for 30 minutes at 4°C, washed again twice with PBS A+B and dried in a stream of warm air. 250 µl of 1 M NaOH was added to each well and the cells digested overnight by placing the trays in a moist plastic box inside an incubator at 37°C. The digest was split into aliquots,

SCHEME 1

β-particle (electron)

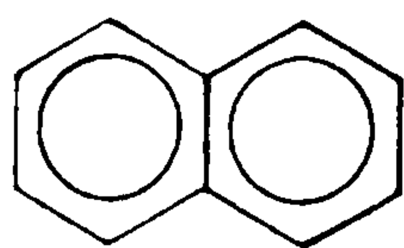
1. Aromatic solvent trapping energy from β-particle, (toluene)



(II)

emission = 285nm

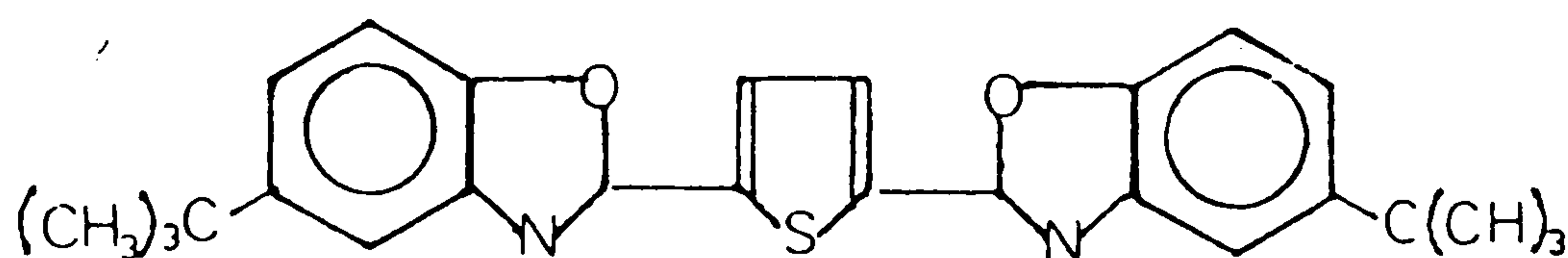
2. Solid which is dissolved in toluene whose excitation energy is similar to that of the fluorescence emission of the solvent itself and which re-emits energy at a longer wavelength, (Napthalene)



(III)

emission = 352 nm

3. Similar to 2. but re-emits energy at a wavelength detectable by the photomultiplier, (called 'wavelength shifters'), 2,5-bis-(5'-tert-butylbenzoxazolyl-[2'])-thiophene (BBOT)



(IV)

emission = 430 nm.

4. Emulsification of the aqueous sample in the organic solvents was assisted by the inclusion of 2-methoxyethanol.

(Horrocks,1970)

100 µl of which was mixed with 5 mls of BBOT scintillation fluid (consisting of 4g/l 2,5-bis-(5'-tert-butylbenzoxazolyl-[2'])-thiophene in a solution composed of toluene/napthalene/2-methoxyethanol 55:8:37) and counted in an Intertechnique SL30 scintillation counter. Estimations were made in quadruplicate. The counting time was set to 10 minutes for each sample. The scintillation counts are proportional to the amount of ^{3}H-TdR incorporated into DNA and is a reflection of the number of cells in S-phase during the labelling period, assuming the rate of DNA synthesis is constant.

The remaining aliquots were used for measurement of the optical extinction at 280 nm which was used as an estimate of the total cellular protein. The extinction at 280 nm was used merely as a check that approximately the same number of cells were present in each well. No correction was found to be necessary for loss of cells in the experiments described. The thymidine incorporation data are expressed as a percentage of the incorporation after treatment compared to untreated controls. These percentages are given as a mean of quadruplicates together with the standard deviation from the mean.

3.2.13 Statistical tests

3.2.13.1 Student's T-test

For all experiments with cells which were set up either 'in triplicates or quadruplicates, a standard student's T-test was used to calculate the standard

deviation and degree of significance. The description of this test together with a modified standard statistics programme is given in Appendix I.

3.2.13.2 Linear regression analysis

This test was used to enable data from several experiments using the same treatments to be summarised and provides information on the reproducibility of the experiment. It is based on the method of least squares. The calculations were made using a microcomputer statistics programme (see Appendix II for details).

3.3 RESULTS

Cells which were exposed to iron complexes were examined for the uptake of iron both qualitatively and quantitatively.

3.3.1 Quantitative estimation of cell-associated iron

The amount of iron internalised by cells incubated with different iron complexes was estimated on detergent lysates. The seeding density used was 5 x 10^4 cells/ml in small Falcons. The medium was removed the following day, the cells washed with PBS A+B and incubated for 2 hours at 37°C with PBSi containing the Fe/complex (100 µM : 500 µM, ie. excess of ligand) previously prepared. The complexes included Fe/8-HQ, Fe/ATP, Fe/EDTA and Fe/dex. Cells were exposed to Fe/dex at a concentration equivalent to 50mM iron for 4-6 hours, as uptake at low concentrations was difficult to detect by this method. The incubation mixture was removed and the cells washed twice with PBS A+B, with 1 mM EDTA to remove external iron, and then twice with PBS A+B. The cells were detached by trypsinisation, resuspended in growth medium and counted by Coulter counter. After centrifugation, the medium was removed and replaced by 1.0 mls of 1% Nonidet P40 detergent (BDH Chemicals). The cells were dipersed in the detergent by Pasteur pipette and left standing for 5 minutes to lyse. At the end of this period 2 mls of 10 mM ascorbate was added to the lysate to reduce the iron to Fe^{2+}. One control tube contained no cells. A second

control tube contained lysate from cells that had not been exposed to any iron complex. The tubes were heated at 60°C for 30 minutes. This produced some turbidity presumably by denaturation of the protein, but this was removed by neutralising the solutions with 1 drop of 1M NaOH. On cooling 2 mls of 5 mM ferrozine was added to detect the reduced iron. After 30 minutes at 37°C the absorbance was read at 562 nm against the blank (no cells). The amount of iron internalised by the cell was estimated by the difference in absorbance between test and untreated controls. The same procedure was carried out with cells which were incubated with iron complexes at 4°C.

The amount of cell-associated iron varied with the nature of the complexing agent. From table 3.1 it is evident that of the ligands tested, iron is taken up by the cells only when complexed to 8-HQ. Uptake also occurred at 4°C to the same extent.

Quantitative estimation of Fe/dex under these conditions was unsatisfactory. However, when cells were incubated for a prolonged period with 50mM Fe/dex considerable complexation to ferrozine from cell lysates was evident (table 3.2). However, in contrast to the uptake of iron in the 8-HQ complex the uptake of Fe/dex was inhibited at 4°C.

In chapter IV the extent of cellular uptake of Fe/complexes is related to their influence on the

TABLE 3.1

IRON CONCENTRATION IN CELLS TREATED WITH DIFFERENT IRON COMPLEXES

Treatment	$[Fe^{2+}(FZ)_3]$ mean (μM)	mean cell no. x 10^6	Total iron fM $cell^{-1}$
control 37°C	1.64	2.6	3
ATP/Fe	2.00	3.7	3
EDTA/Fe	1.68	1.9	4
HQ/Fe	5.10	1.5	17
HQ/Fe 4°C	3.20	1.0	16

* Initial iron concentration = 100μM
Initial Ligand concentration = 500mM
Attached cells were incubated in flasks in PBSi with the iron complex for 2 hours.

TABLE 3.2

FERROZINE-DETECTABLE IRON FROM CELLS TREATED WITH IRON DEXTRAN

Incubation with Fe/dex (50mM)	T°C	mean concⁿ (μM)*	mean cell no. x 10^6	fM $cell^{-1}$
control	37	8	1.8	22
BM 324s	37	25	1.6	78
control	4	8	1.14	35
BM 324s	4	9	1.3	35

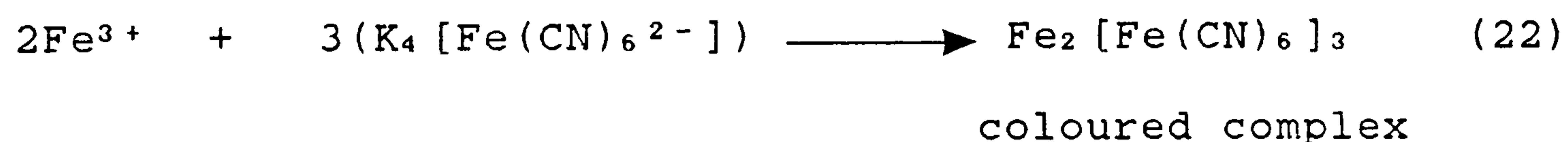
Attached cells in flasks were incubated with Fe/dex for 4-6 hours in growth medium.

* of $[Fe^{2+}(FZ)_3]$ complex

cytotoxic action of H_2O_2.

3.3.2 Staining for iron (prussian blue)

The localization of iron taken up by cells could be observed using staining methods for either reduced or oxidised iron. For Fe^{3+} Prussian Blue, first described by Perls (1867), was used. The method was essentially as described by Lison (1936) and Bunting (1949) and is summarized by Pearse (1972). This method depends on the production of ferric ferrocyanide when ferric ions react with ferrocyanide in acid solution:



$$2Fe^{3+} \quad + \quad 3(K_4[Fe(CN)_6{}^{2-}]) \longrightarrow Fe_2[Fe(CN)_6]_3 \qquad (22)$$

coloured complex

The procedure was as follows; Cells were inoculated onto a glass microscope slide at a density of approximately 5 x 10^4 cells/ml and incubated at 37°C for 2-3 days. Iron comlexes with an iron to ligand ratio of 1:5 were then added for 2 hours at a concentration of 100 μM iron except in the case of iron dextran where the concentration of iron was 10 mM and the cells were incubated for 24 hours. The cultures were washed with PBS A+B and EDTA and the cells fixed in 4% buffered glutaraldehyde for 10 minutes at 4°C and washed with distilled water. A mixture of 2% potassium ferrocyanide (SIGMA) and 2% HCl prepared immediately before use was

added to the slides for 30-60 minutes. When stained, the slides were dehydrated with increasing concentrations of alcohol, cleared with xylene and mounted with Depex mounting medium.

Of the Fe/complexes tested only cells exposed to Fe/dex and Fe/8-HQ showed significant staining (Plates I and II). Plate IA & B illustrate the staining of cells treated with Fe/8-HQ for 1 hour, showing a general distribution of the stain in the cytoplasm. This is also evident in cells treated with Fe/8-HQ at 4°C. No staining was observed in cells treated with Fe/ATP, Fe/EDTA or Fe/phen.

Cells treated with Fe/dex (10 mM Fe for 24 hours at 37°C) show that the staining is mainly associated with the periphery of the cells and phase contrast pictures show that the stain is associated with large vesicles (plates II & III). Interference contrast images indicated that the stain is concentrated mainly at the periphery of these vesicles (plates IV A).

When cells were exposed to Fe/dex at 4°C vesicles were absent and no iron staining was observed. The cells resembled those of the untreated controls at 37°C and 4°C; (plates III C & D).

3.3.3 Staining for dextran

As iron dextrans are large molecular weight compounds, the dextran component should be detectable by staining methods if it enters the cell. Initial attemps

Legend to Plates:

Magnifications on prints:
Objectives 25 or 63 or 100
optavar x 1.25
projection lens x 3.2
neg 35mm, print 125mm
total x 14.4

PLATE I: Arrows show both peripheral and general cytoplasmic staining at 37°C and 4°C.

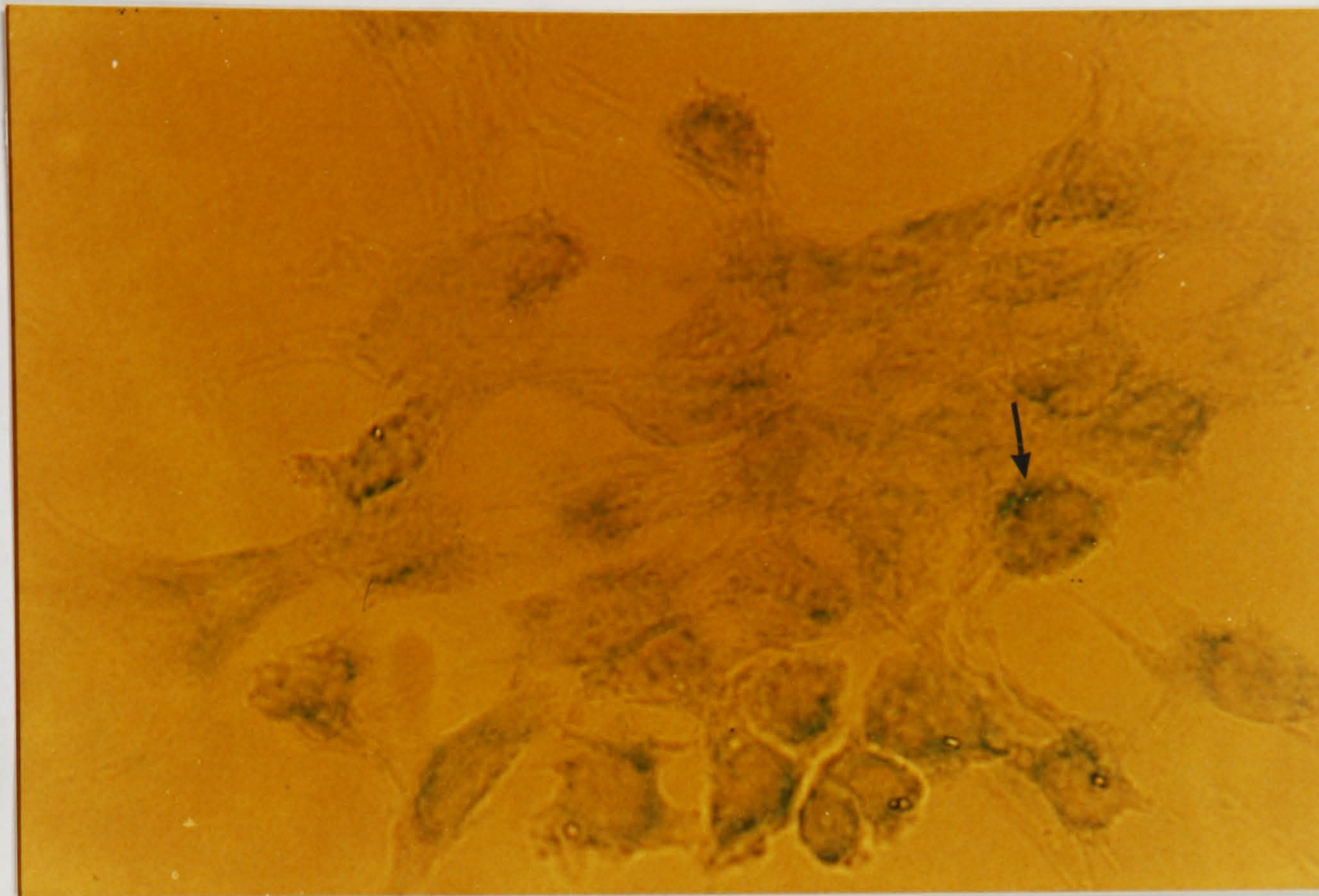
PLATE II: Arrows point to vesicular staining associated with Fe/dex uptake.

PLATE III: B is a direct transmitted image of phase contrast A. Arrows on plate point to vesicular staining.

PLATE IV:
A: Arrows point to stain associated with periphery of vesicles.
B: Arrows point to peripheral flourescence, presumed to be intact dextran as distinct from general backround.
Exposure to UV: 4mins.

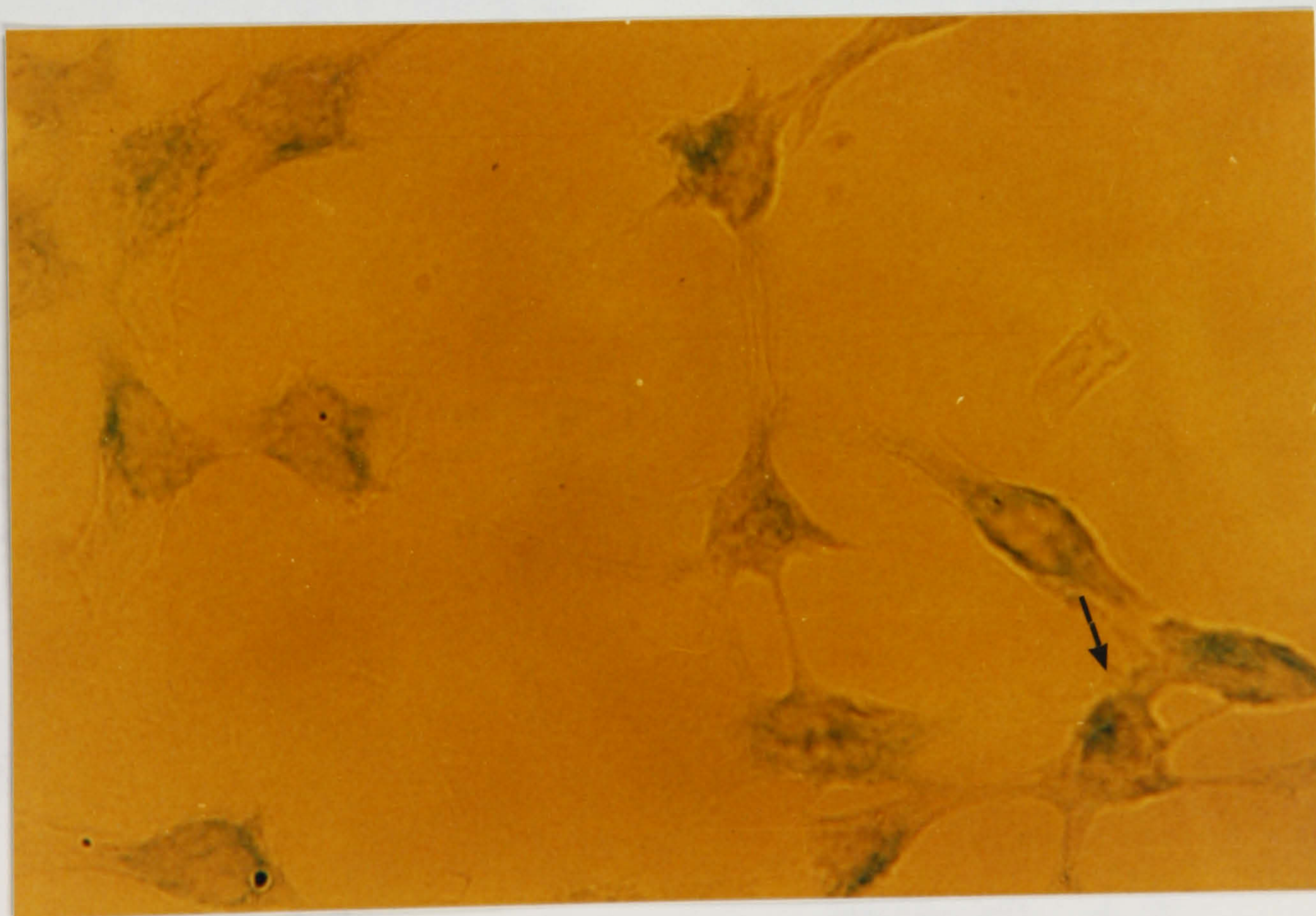
PLATE I

A



Fe/8-HQ 37 °C 1hr in PBSi

B

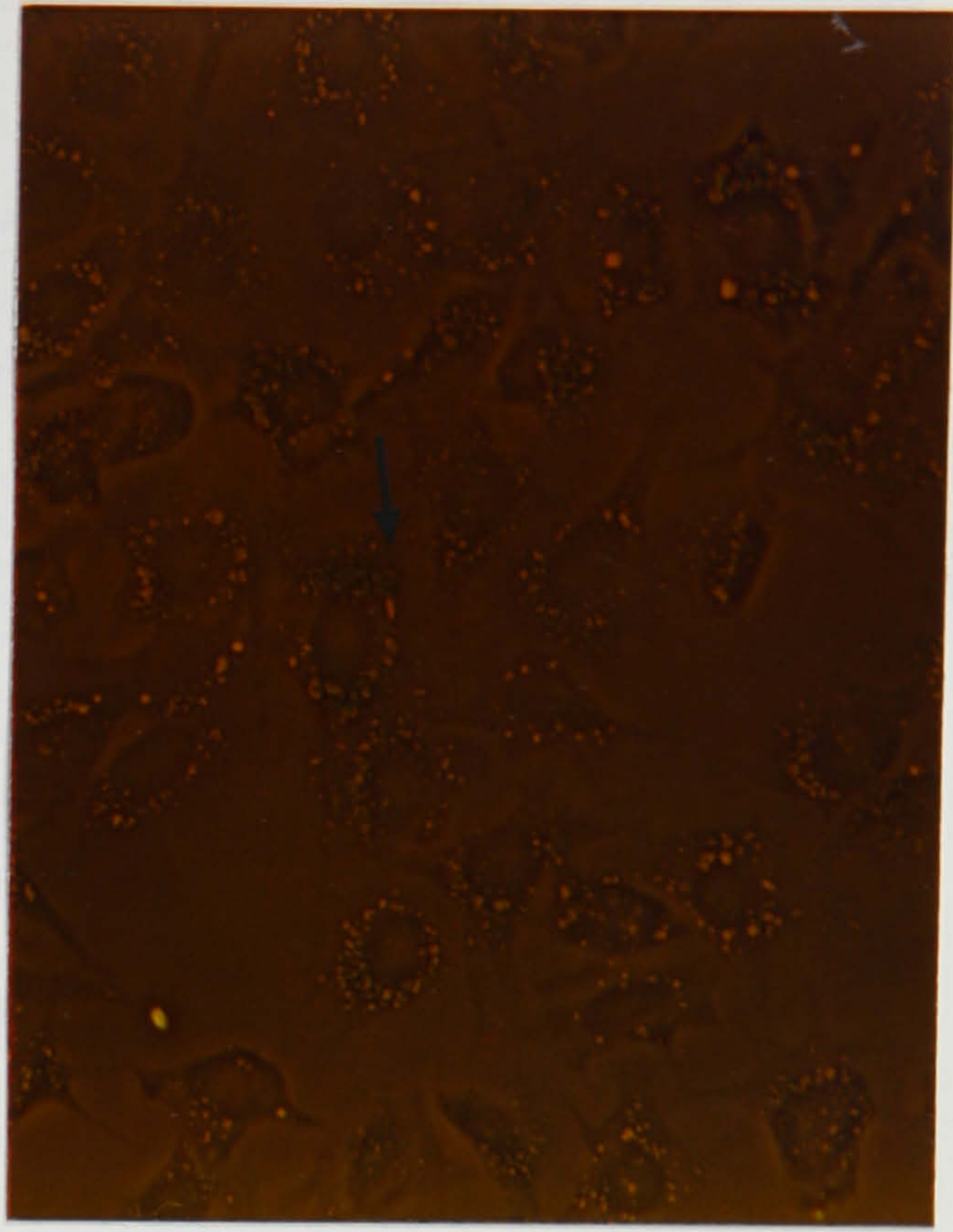


Fe/8-HQ 4 °C 1hr in PBSi

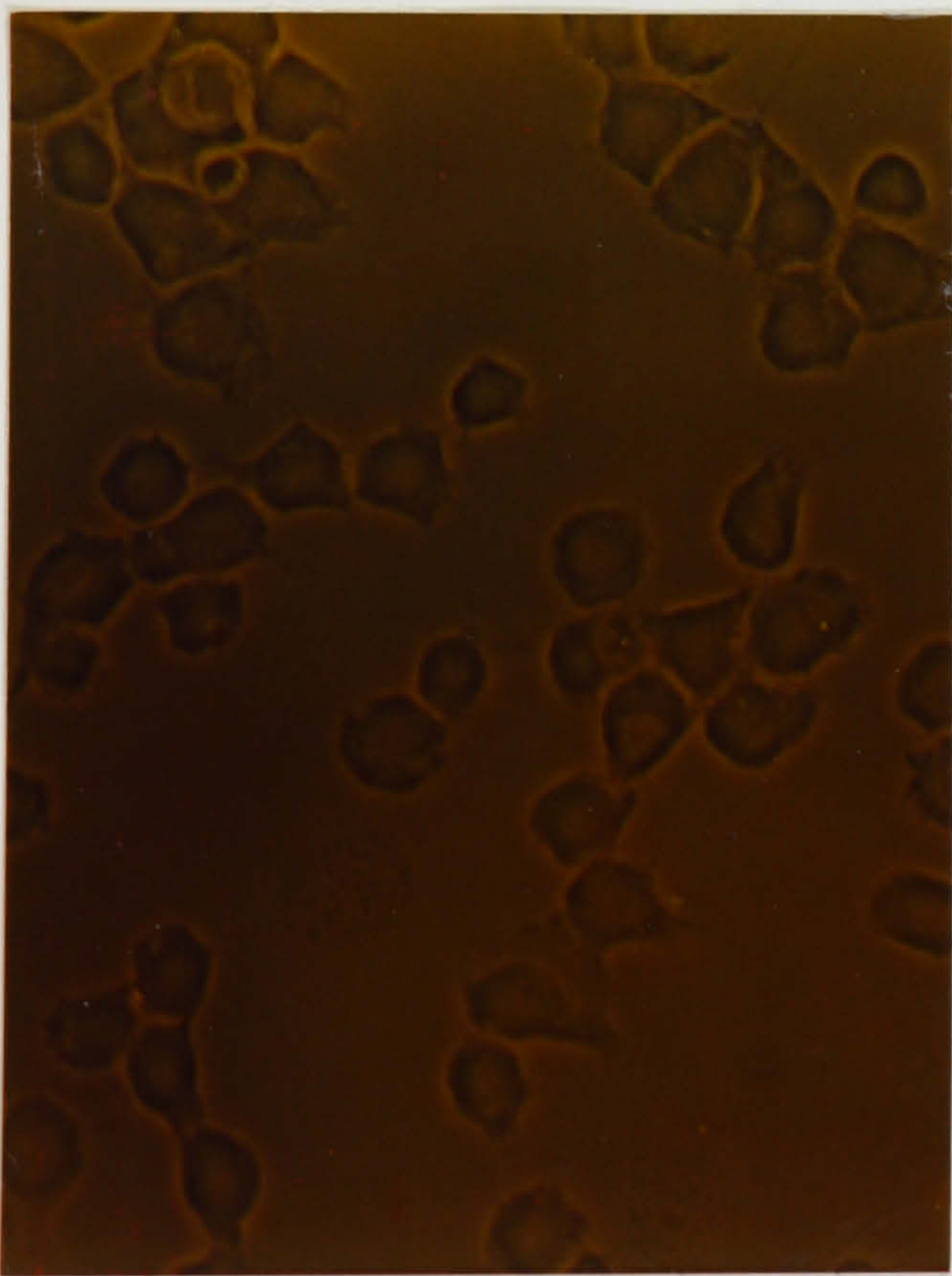
direct transnmitted light
magnification on print 900x

PLATE II

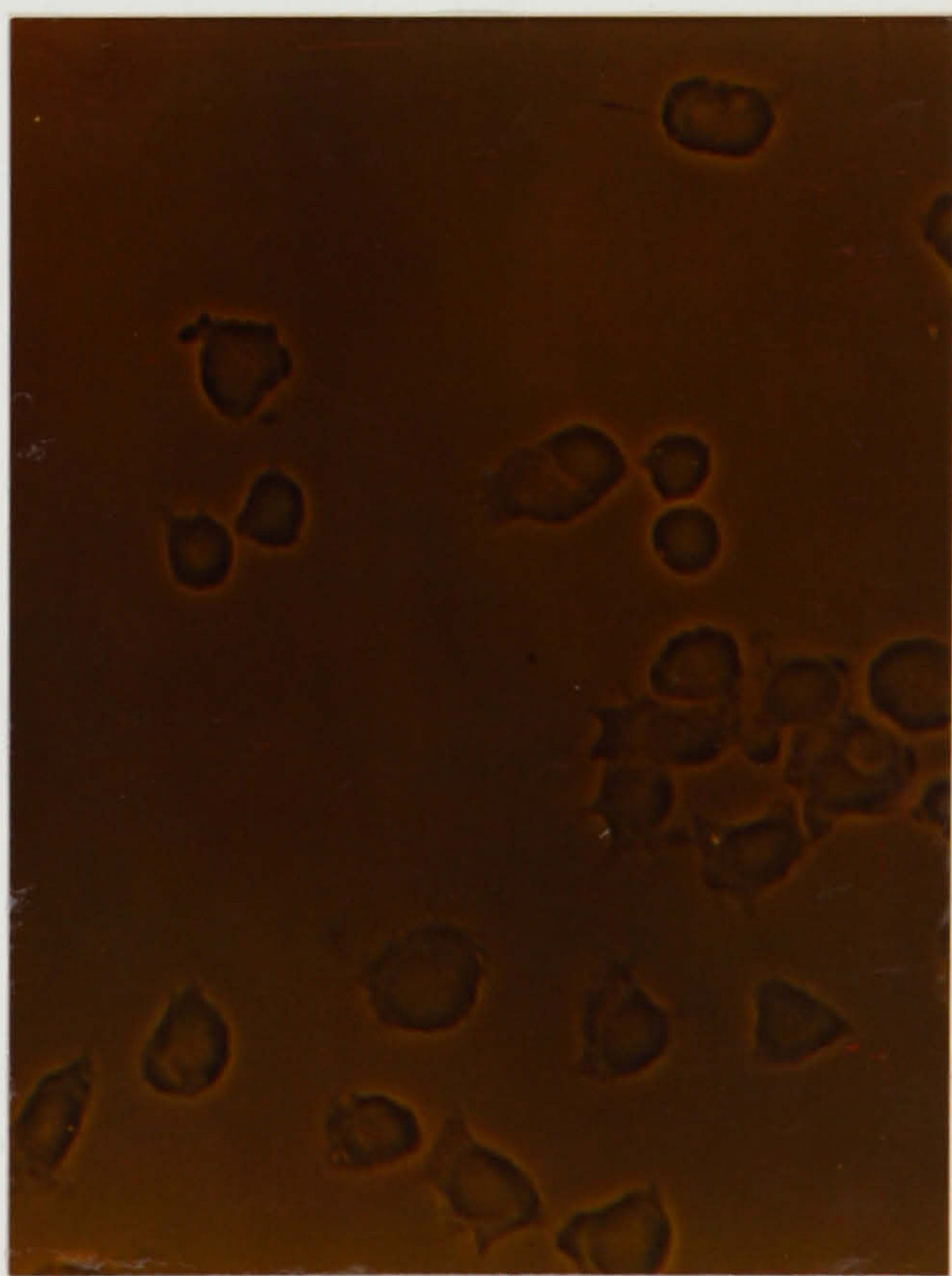
control 37 °C



Imferon 37 C



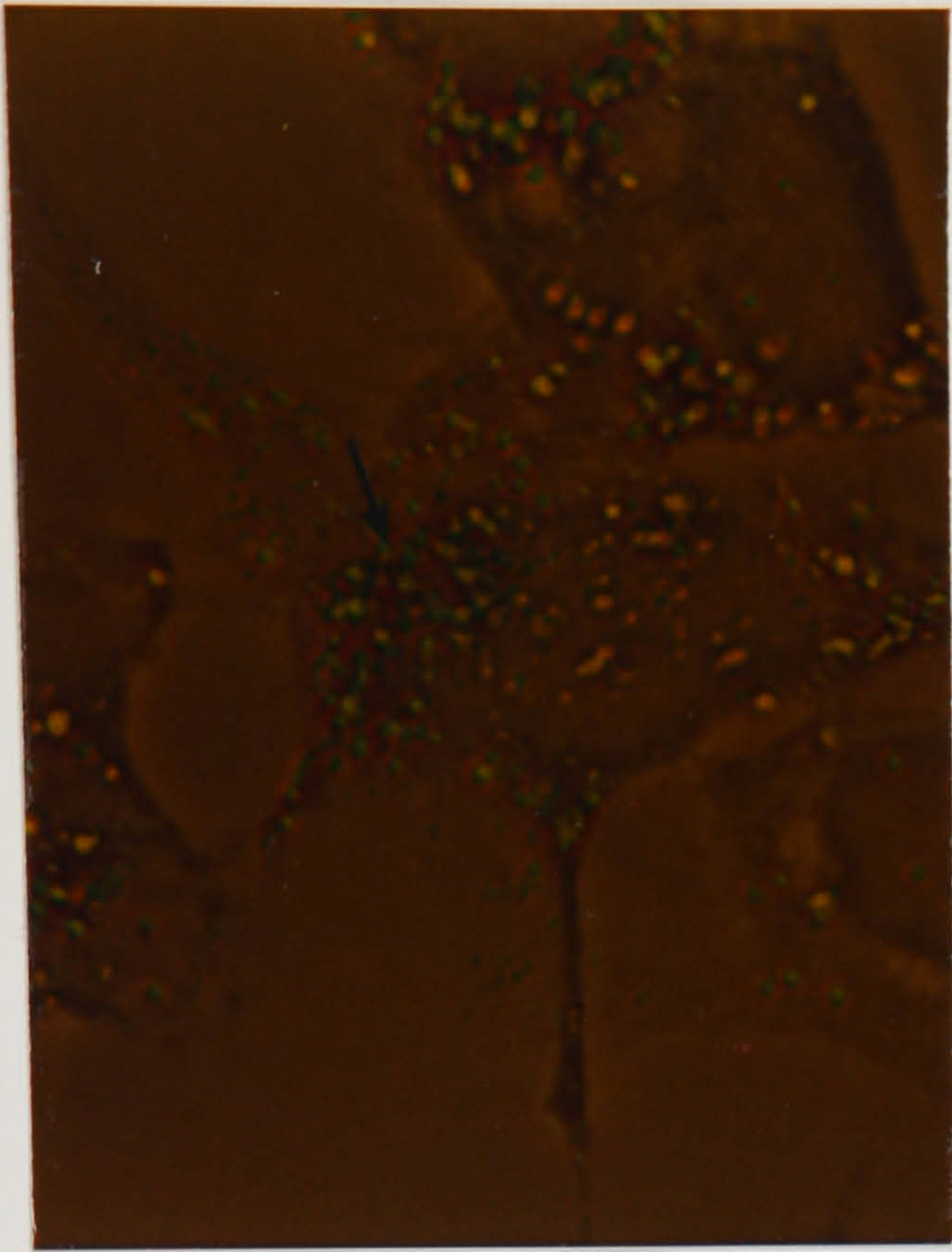
control 4 °C

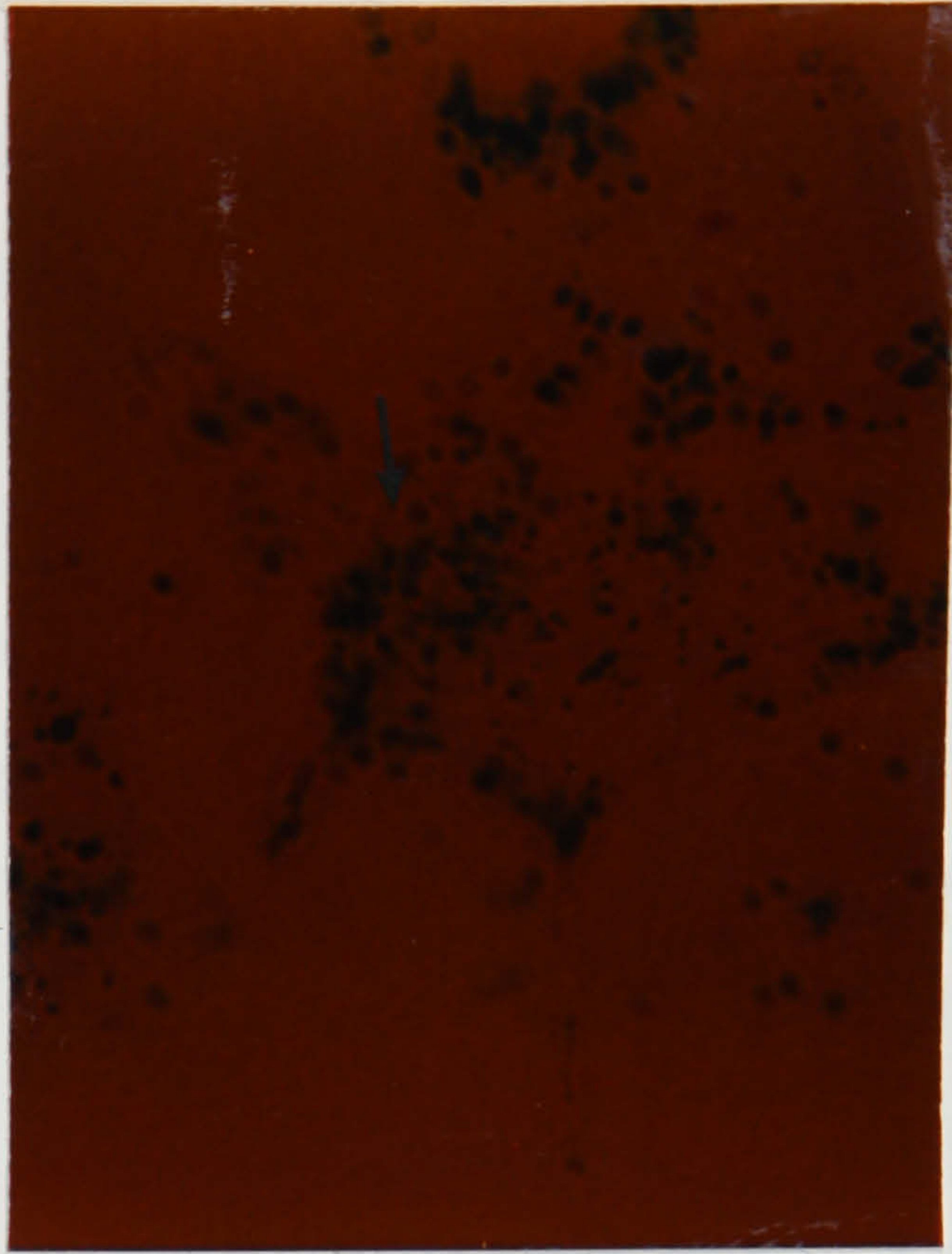


Imferon 4 C

all phase contrast   magnification on print 360x

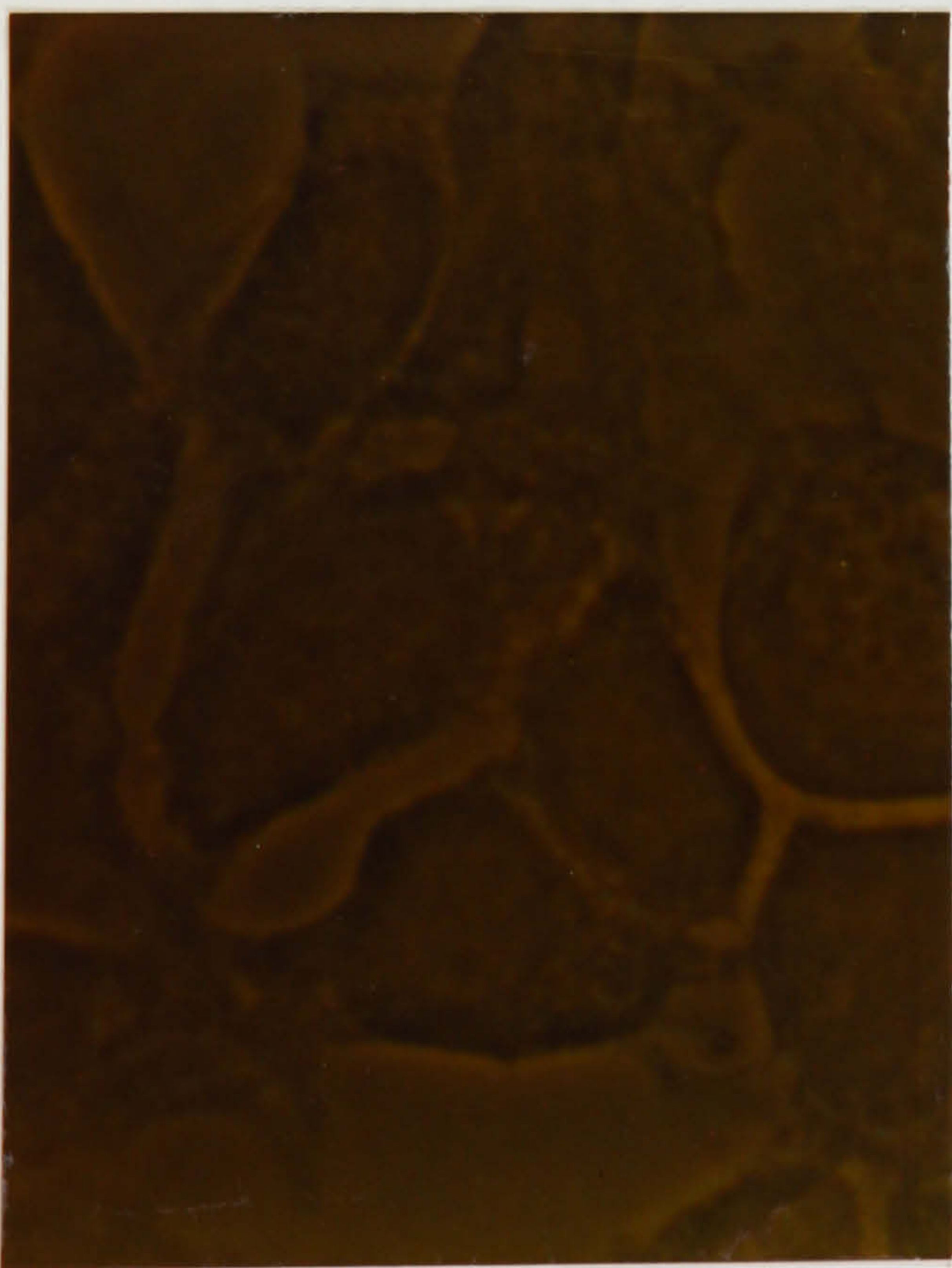
PLATE III

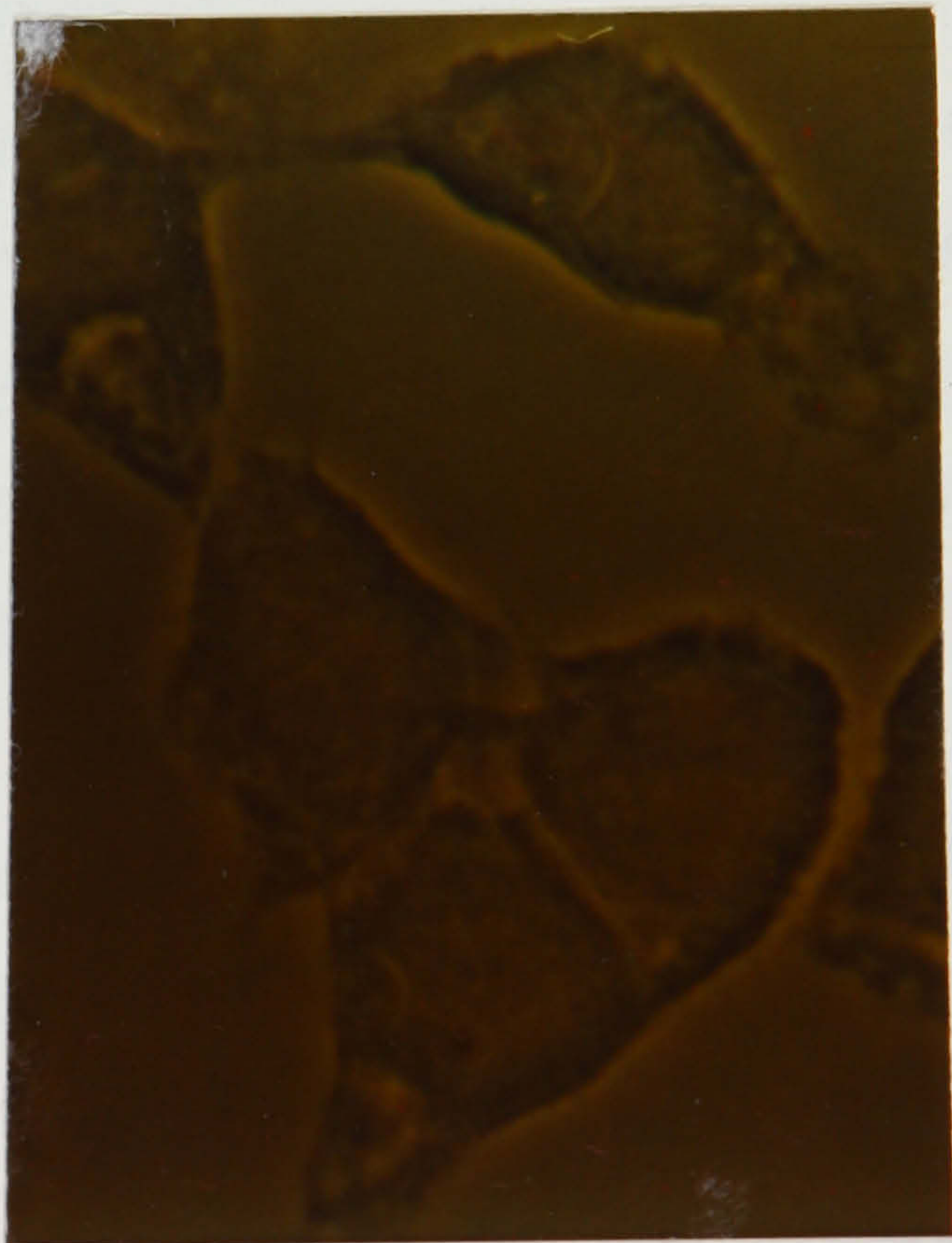




Imferon 37 °C A

Imferon 37 °C B





control 4 °C C

Imferon 4 °C D

ACD phase contrast
B direct transmitted light
magnification on print 1430x

were made to stain for the dextran component of Imferon using the periodic acid-Schiff (PAS) procedure as a carbohydrate-specific staining method. Schiff reagent is a basic dye (Fuchsin) which has been rendered colourless by addition of aqueous SO_2 which results in loss of the quinoid structure and consequent loss of colour; in the presence of aldehydes the quinoid structure is restored. Periodic acid oxidises carbohydrates to generate aldehydes which react with Schiff's reagent. The technique described by Culling (1974) was used. However, staining in both treated cells and untreated controls was very intense indicating that these cells contain high levels of carbohydrate material which prevented the detection of uptake of dextran.

An alternative technique using fluorescent dextran of similar molecular weight to Imferon was employed. To correspond as closely as possible to the 180 KDa molecular weight of dextrans in iron dextran complexes, a 150 KDa fluorescein isothiocyanate-linked dextran (FITC-dextran) was used.

A FITC-dextran solution was prepared and was added to the medium of cells at a final concentration of 0.2% on a slide for 24 hours. Cells were washed, fixed with 4% glutaraldehyde and dried in air. A non-fluorescent glycerin mounting medium was used (neutral glycerin jelly, Raymond A. Lamb) to mount the cells and the slide examined for fluorescence on an UV microscope with

appropriate filters.

Plate IV B shows some cells with circumscribed peripheral zones of strong fluorescence which may represent internalised dextran associated with vesicular compartments of the cell. All cells exhibited a general backround level of fluorescent staining. This may be the result of rapid breakdown of dextran by glycosidases of the cell, with release of flourescein into the cytosol.

3.3.4 Effects of iron complexes on cell survival

The cells were incubated with various ligands in the presence or absence of iron.

3.3.4.1 ^{3}H-TdR incorporation

Survival was assessed using the ^{3}H-TdR incorporation assay. In these experiments imidazole buffer (0.1M) pH 7.40 was used containing Ca^{2+} and Mg^{2+} salts in the same concentrations as for PBSi. The cells were seeded at 5 x 10^{4} cells/ml and used the following day. The cells were washed with PBS A+B and incubated for 1 hour at 37°C with Imidazole buffer containing the ligand with or without $FeSO_4$. Iron complexes were prepared immediately before addition to cells. $FeSO_4$ was added to the cells at a final concentration 100 μM with 500 μM of the ligand. ^{3}H-TdR incorporation was assessed immediately after the incubation.

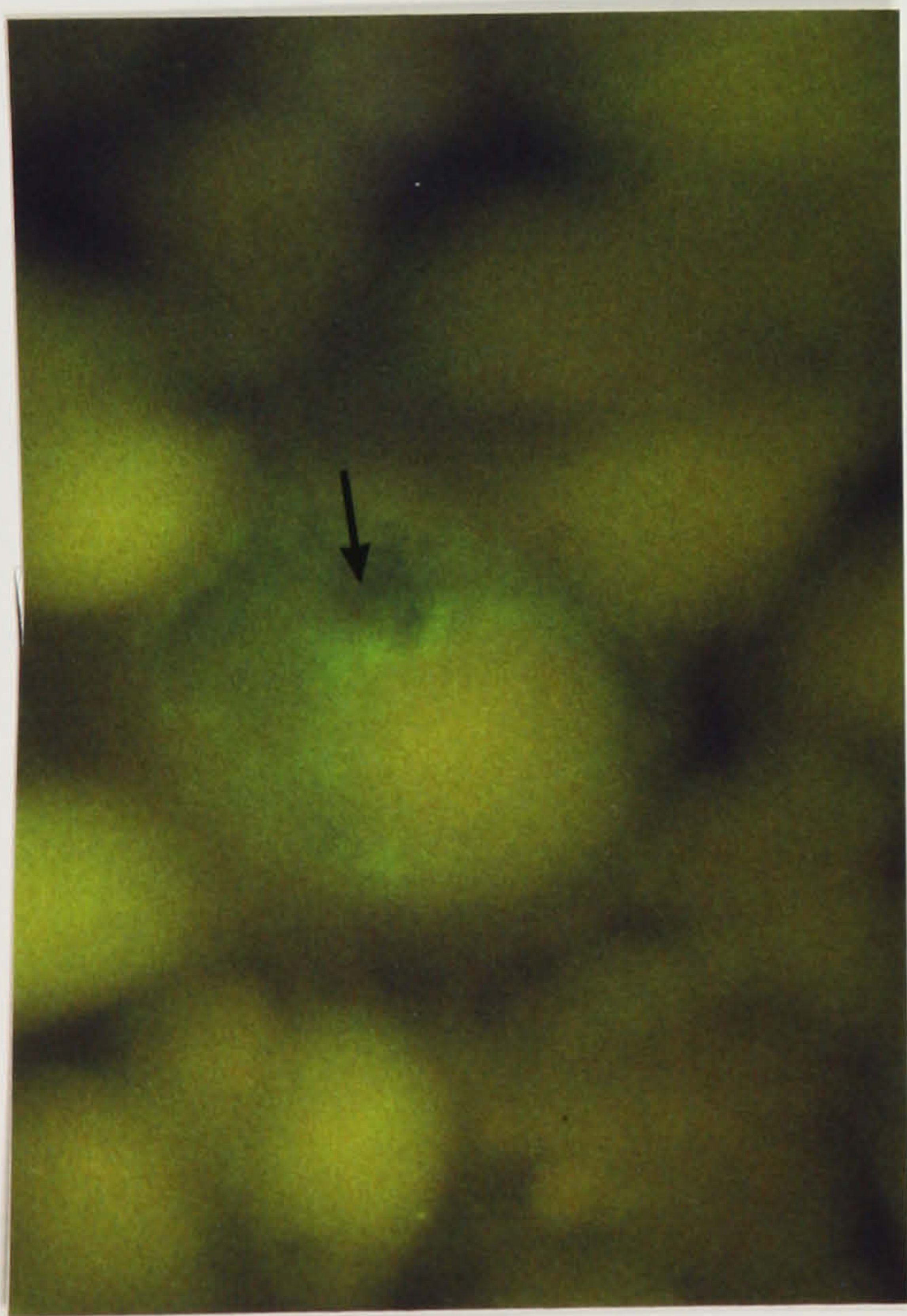
The effect of 8-HQ was studied with both Fe(II) and Fe(III). In this case PBSi was the buffer used. Cells were incubated in multiwells with 100 μM of either $FeCl_3$

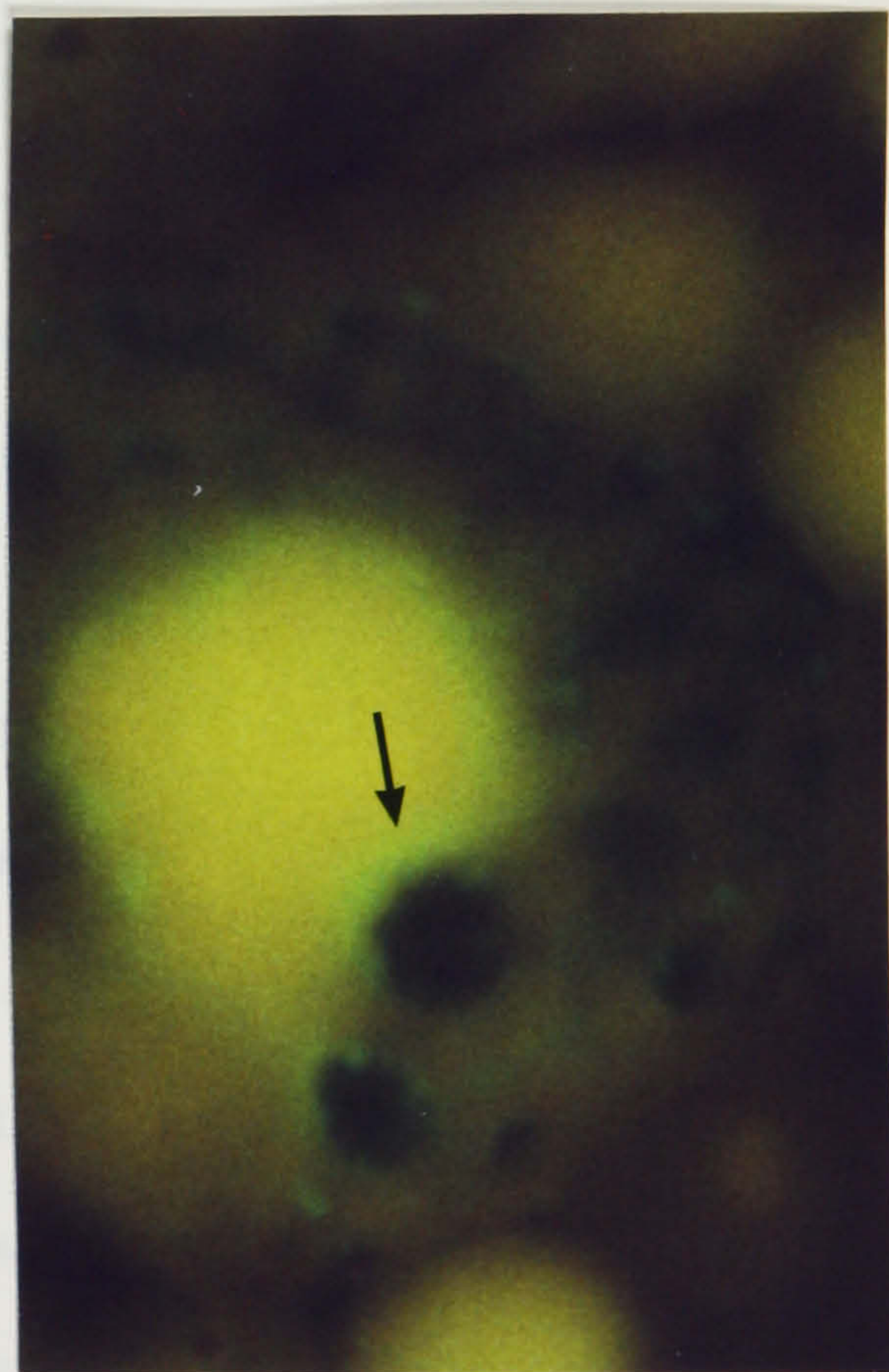
PLATE IV

A

Imferon Interfernce contrast

B





FITC-dextran UV

magnification on print 1430x

or $FeSO_4$ and increasing concentrations of 8-HQ. Controls were incubated with 8-HQ in the absence of iron.

All the ligands (except dextran C), tested in the absence of any iron had some effect on thymidine incorporation by the cells. Since the studies on incorporation of ^3H-TdR were carried out immediately following exposure to the agents the reduced incorporation reflects an immediate effect which is most likely to be due to the detachment of cells. This was confirmed by direct observation and OD_{280} readings which give an estimate of the total number of cells in the wells. In the presence of $FeSO_4$ the inhibitory effect of these ligands on the incorporation of ^3H-thymidine was considerably reduced except in the case of DTPA, where there was no significant difference, and 8-HQ which is highly toxic in the presence of $FeSO_4$. These results are shown in table 3.3. In the case of Fe/8-HQ toxicity the damage was visually evident from membrane blebbing and general cell disintegration.

Increasing concentrations of 8-HQ showed relatively little toxicity in the absence of iron (fig 3.1). However, in the presence of either $FeSO_4$ or $FeCl_3$ (100 μM) substantial toxicity is observed from 200μM of 8-HQ. The results are consistent with studies by Rubbo _et al_. (1950) and Albert _et al_. (1953) who showed the necessity of a ' co-toxicant metal for the bactericidal or bacteriostatic action of 8-HQ. The results for 8-HQ alone

TABLE 3.3

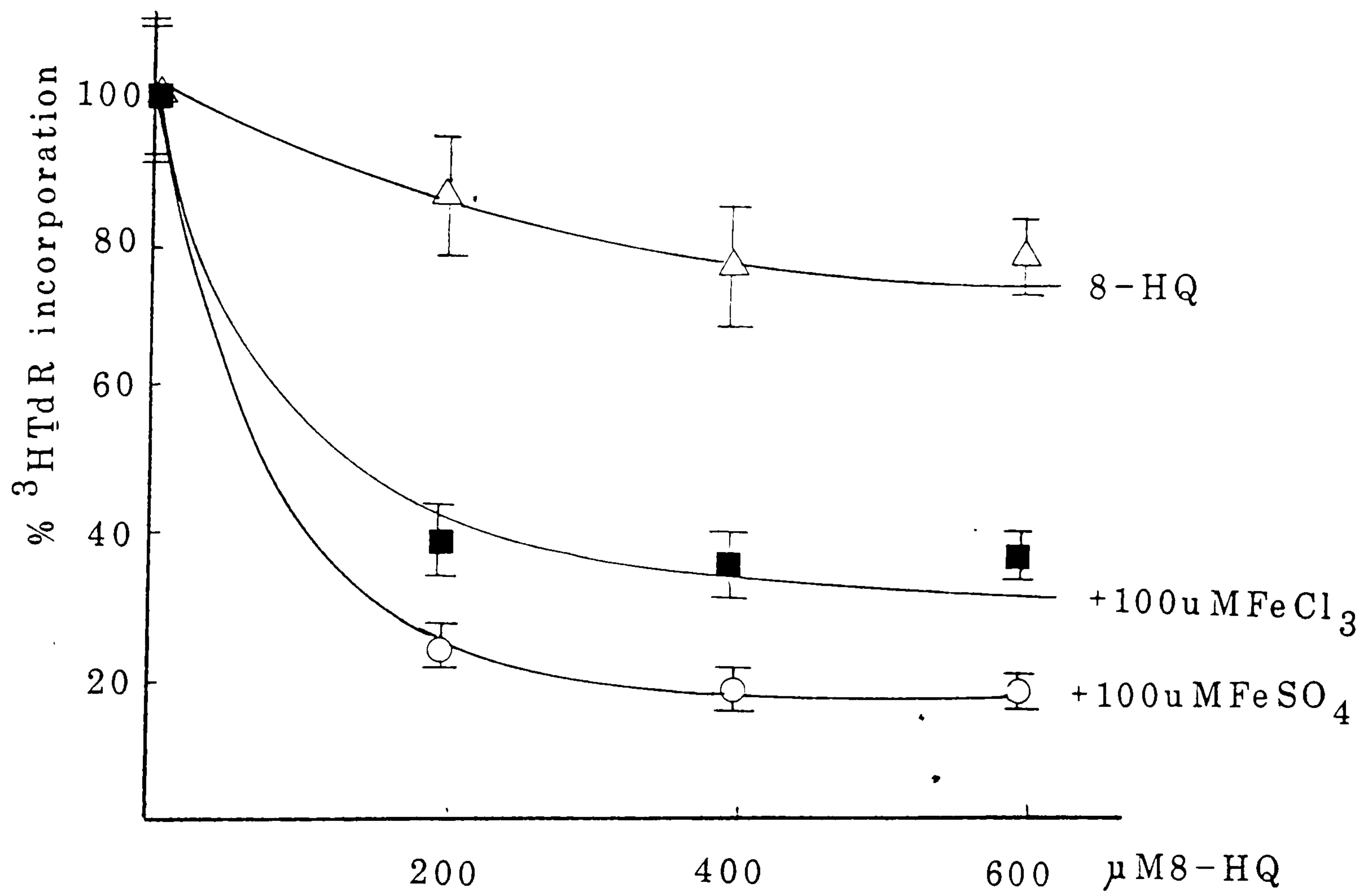
EFFECT OF LIGANDS IN THE PRESENCE AND ABSENCE OF $FeSO_4$ ON ^{3}H-TdR INCORPORATION

Ligand 500µM	$FeSO_4$ 100µM	%^{3}H-TdR incorporation (cpm)	%P (a)	%P (b)
-	-	100 ± 4		
-	+	110 ± 3		
phenan-throline	-	48 ± 5	<0.01	0.313
	+	80 ± 10	1.82	
ATP	-	56 ± 5	<0.01	2.78
"	+	70 ± 7	0.11	
ADP	-	62 ± 6	0.03	n.s.
"	+	72 ± 12	0.901	
8-HQ	-	61 ± 3	0.01	<0.005
"	+	8 ± 1	<0.005	
DTPA	-	50 ± 3	0.01	n.s.
"	+	44 ± 10	0.03	

(a)= probability value with respect to untreated control;
(b)= probability value compared to effect of ligand alone

EFFECT OF 8-HQ ON CELL VIABILITY

IN THE PRESENCE OF Fe SALTS



$1-2 \times 10^5$ c/ml

PBSi pH7.40

incubation time 1hr

fig3.1

given in table 3.3 and fig 3.1 are dissimilar. This discrepancy may be due to the incubation medium used, ie imidazole buffer and phosphate buffer respectively.

3.3.4.2 Plating efficiency

The survival of cells exposed to iron complexes in which the ratio of iron to ligand was 1:1 was estimated by plating efficiency. Suspensions of 5 x 10^4 cells/ml were incubated for 1 hour with the complex (100μM) in PBSi at 37°C. Plating efficiency was carried out as described in section 3.2.11.

Cell survival was not significantly affected by the addition of any Fe/complex in a 1:1 ratio. (table 3.4). The difference between these results and those presented in table 3.3 can be accounted for by the ratio of ligand to iron, which is 1:1 in table 3.4 and 5:1 in table 3.3. Thus there is an excess of ligand in one case which can inhibit the incorporation of thymidine to some extent.

3.3.4.2.1 Comparison of batches of iron dextran

A range of Fe/dextrans were tested at different concentrations to examine possible evidence of inter-batch variation. Freshly opened samples after appropriate dilution were added directly to the cells which were incubated in PBSi at 37°C for 1 or 2 hours and plating efficiency carried out as described above, (3.3.4.2).

No toxic effect of Fe/dextrans was seen for any of the batches tested up to millimolar concentrations and

TABLE 3.4

SURVIVAL OF CELLS IN THE PRESENCE OF IRON COMPLEXES

Complex (100μM)	% survival P.E.
Fe/phen.*	103 ± 10
Fe/EDTA	97 ± 8
Fe/ATP	96 ± 4
Fe/ATP	109 ± 3

* Fe : phenanthroline 1:3

other iron:ligand 1:1

5 x 10^4 c/ml in suspension were incubated with iron complexes in PBSi for 1 hour.

there was no evidence of inter-batch variation. This correlates with thymidine incorporation in which no inhibitory effect was evident for the same concentrations used.

3.4 DISCUSSION

The inhibition of ^{3}H-TdR incorporation by unbound ligand is indicative of a transient cell detachment. This results in a lower spread area of the cells and reduced DNA synthesis. The degree of detachment is partially reduced upon the addition of iron because chelation of Ca^{2+} necessary for cell adherence is reduced. However, as the ligand to iron ratio was 5:1 a limited degree of detachment could have been caused by the excess of unbound ligand. This may account for the lack of an equivalent effect on P.E., where the ligand was not present in excess of iron. For example, in the case of phenanthroline which forms a 3:1 complex with Fe(II), ^{3}H-TdR incorporation was increased from 48% to 80% upon addition of 100μM $FeSO_4$, ie 200μM excess ligand inhibited by 20%. The corresponding P.E. results show a 100% survival when no excess of ligand is present.

8-HQ is the only ligand that showed significant increase in inhibition in 3H-TdR incorporation upon addition of Fe (61% to 8% in imidazole buffer or 80% to 20% in phosphate buffer). This correlates with partition coefficients and suggests that lipophilicity is necessary for a complex to enter the cell and that all other compounds tested remain extracellular. This was confirmed by iron staining in which only Fe/8-HQ and Fe/dex showed significant staining.

Fe/dex is not lipophilic but enters the cell by

endocytosis. The process was inhibited at 4°C whereas no impairement to the passive diffusion of Fe/8-HQ was evident. Further evidence for uptake of iron in these two cases was provided by analyses of cell lysates.

Evidence from FITC-dextran suggests that the dextran is rapidly degraded in the vesicular fraction (presumably by lysosomal glycosidases) with release of fluorescein which enters the cytosol. However, cytochemical staining (plates) indicates that the iron released by dextran breakdown is retained in the vesicular compartment.

Endocytic vesicles, once fused with primary lysosomes, contain a range of different enzymes capable of hydrolytic splitting (Pitt, 1975) which may be responsible for the release of iron from the dextran.

The inhibition by Fe/8-HQ is consistent with its cytotoxic action, a property which has been exploited in its use as a strong fungicidal and bactericidal agent. Its mode of action has been studied by Albert since 1947 (Rubbo, 1950; Albert, 1953).

No toxic effects were ascribed to any batches of Fe/dex, despite evidence of uptake by cells.

CHAPTER IV: STUDIES INTO THE EFFECT OF HYDROGEN PEROXIDE ON MAMMALIAN CELLS

4.1 INTODUCTION

The intracellular levels of H_2O_2 are, under normal circumstances, controlled by H_2O_2-removing enzymes. These include principally catalase and glutathione peroxidase.

(a) Glutathione peroxidase (GPx)

Glutathione peroxidase catalyses the reaction:

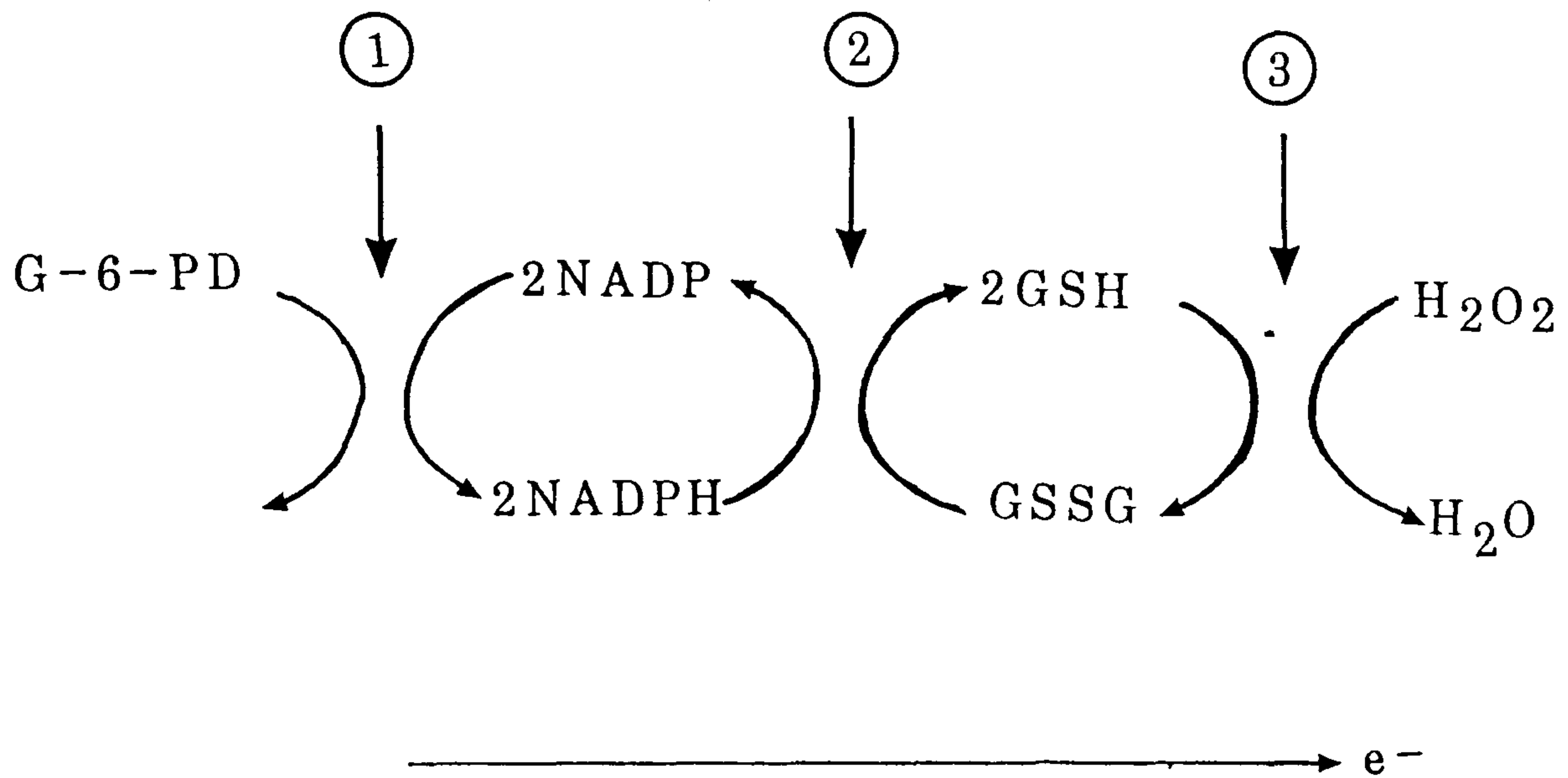
$$H_2O_2 + 2GSH \longrightarrow GSSG + H_2O \qquad (23)$$

Oxidised glutathione is reduced by glutathione reductase which uses NADPH as the electron donor. The reducing equivalents for GSH are mostly derived from NADPH generated by the hexose monophosphate shunt. A scheme of the coupling of H_2O_2 reduction to the pentose phosphate shunt is illustrated in fig. 4.1. The removal of H_2O_2 by this pathway is thus dependent on the efficiency of the generation of reducing equivalents by the cell. The reducing equivalents are ultimately supplied by glucose, but the activity of GPx may be limited by the availability of GSH. In most epithelial cells the concentration of GSH is in the region of 25-40 nmoles/10^6 cells (Jones et al.,1981).

(b) Catalase

In contrast to GPx, which is distributed in the cytosol and mitochondrial matrix space (70% and 30%

<u>METABOLIC INTEGRATION OF CATABOLISM OF H_2O_2 BY GPx</u>



① HEXOSE MONOPHOSPHATE SHUNT INVOLVES REACTIONS CATALYSED BY GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND 6-PHOSPHOGLUCONATE DEHYDROGENASE GENERATING 2NADPH.

② GLUTATHIONE REDUCTASE

③ GLUTATHIONE PEROXIDASE

fig 4.1

respectively in rat liver, Flohe,1971), catalase is mainly restricted to the peroxisomes of the cell (Lazarow & de Duve, 1973) although some conflicting reports suggest the possibility of extraperoxisomal catalase due to the fragility of the peroxisomes (Holmes & Masters,1972; Kashiwagi et al.,1971). It catalyses the reaction:

$$2H_2O_2 \longrightarrow O_2 + 2H_2O \qquad (24)$$

and does not require a second substrate. The reaction involves redox changes in the porphyrin iron of the enzyme and the reaction appears to proceed by a number of intermediate steps involving the porphyrin-bound iron (Chance,1949; Schonbaum & Chance, 1976).

The reaction rates of both GPx and catalase are very similar; that of GPx has been determined as approximately 2×10^8 M^{-1} s^{-1} (Flohe et al.,1972) and of catalase as 3.5×10^7 M^{-1} s^{-1} (Chance et al.,1952). The relative contribution of these major pathways in removing H_2O_2 is dependent on the concentration of H_2O_2. In cases where the H_2O_2 concentration is low GPx is the predominant system. Several authors have shown in systems where H_2O_2 is generated by xanthine oxidation catalase is the major contributor to the degradation of H_2O_2 (Jones & Kennedy,1983; Hirashi et al.,1987; Bulkley et al.,1987). Catalase is ineffective at low concentrations of H_2O_2.

Its Km has been reported as 1.1M (Nicholls & Schonbaum,1963).

When the capacity of these metabolising enzymes is exceeded H_2O_2 becomes toxic.

There is now considerable evidence that the hydroxyl radicals from water radiolysis are a major cause of gamma-radiation-induced cell killing (Johansen & Flanders, 1965; Roots & Okad, 1975; Chapman et al., 1973). Lesions of DNA produced by H_2O_2 are similar to the damage produced by HO· radicals from ionising radiation (Ward,1975), although the yields differ considerably (Elkind & Redpath,1977). It has been proposed that hydroxyl radicals are formed by metal ion catalysed reduction of H_2O_2. The possible involvement of metals in damage to DNA is indicated by the fact that no SSB occurred in purified DNA which had been previously dialysed with EDTA (Menighini & Hoffmann,1980). The toxicity of H_2O_2 occurs in the presence of reduced iron associated with nuclear proteins or DNA (Zimm & Le Bret,1983). SSB induced by H_2O_2 were found to be inhibited by the addition of the metal chelators phenanthroline or bipyridyl (Mello Filho & Meneghini, 1983). In a recent study by Larramendy et al. (1987) using chinese hamster fibroblasts, sister-chromatid exchanges, which were formed in the presence of H_2O_2 in a dose-dependent manner, could be inhibited by the addition of phenanthroline to the medium. If the transition metal

with which H_2O_2 reacts is closely associated with DNA the HO· radicals formed, which have a diffusion range of less than 10-20 Å, would be able to react with deoxyribose or the base moieties (Ward et al., 1975).

Conflicting data exist concerning the type of damage to DNA caused by H_2O_2 which leads to cell killing. Ward et al. (1987) presented data showing that a limited number of DSB and no SSB were detectable following exposure of cells to H_2O_2 up to concentrations of 10mM. Exposure to H_2O_2 at 0°C resulted in numerous SSBs but little cell killing. This was associated with a low DNA repair rate at low temperature. No correlation of DNA DSB with cytotoxicity of H_2O_2 at 37°C was observed which resulted in the consideration of additional types of irreversible damage either directly to DNA or to other sensitive sites in the cell.

The work reported here describes the cytotoxic effect of H_2O_2 on I-221 cells and the influence of some agents that modify the cellular response.

4.2 MATERIALS AND METHODS

4.2.1 Reducing agents

4.2.1.1 Ascorbic acid

With cells the agents used were L-ascorbic acid (free acid, SIGMA) and the oxidised form, dehydroascorbate (DHA, Koch-Light Laboratories Ltd.). L-ascorbic acid and DHA was dissolved in phosphate buffer immediately before addition to cells. The pH was adjusted immediately before use and the solutions filter sterilised.

4.2.1.2 Lipoic acid

Reduced lipoic acid (DL,1,2dithiolane-3-pentoic acid, SIGMA) and its oxidised form were obtained from Sigma. Stock solutions were prepared by dissolving in 50% ethanol.

4.2.2 Zinc salts

Zinc acetate, zinc histidine, zinc aspartate and zinc orotate were kindly provided by Professor G.L. Floersheim (Laboratory for Transplantation Biology, Department of Surgery and Research, Zentrum für Lehre und Forschung, Kantonsspital, Hebelstrasse 20,4031, Basel, Switzerland). All salts, except zinc aspartate were white solids. Zinc aspartate (Zn-bis-(DL-hydrogen aspartate), Dr. F. Köhler Chemie GMBH, Alsbach-Bergstrasse) was provided in sealed ampoules in solution at a concentration of 9 mM Zn^{2+} . Solvation of zinc salts was only possible at low concentrations and at pH up to and

including 6.0. At concentrations above 10mM precipitation was found to occur above pH 6.

4.2.3 Cytotoxicity assays

The cytotoxicity of H_2O_2 was assessed by 2 different methods;

(1) plating efficiency (colony formation) and (2) incorporation of tritiated thymidine 24 hours after exposure to H_2O_2.

The two assay systems were compared by a dose-response curve with H_2O_2. In most of the experiments involving H_2O_2 and iron cytotoxicity was assessed using ^{3}H-TdR incorporation after 24 hours.

4.2.3.1 Exposure of cells to H_2O_2 at different temperatures

With the use of a Techmation minifreeze apparatus (model A-80), which controls the rate of change of temperature, a temperature gradient experiment was performed. The temperature of the experimental system was monitored using a sensitive thermocouple. Small petri dishes of 3 cm diameter were seeded with 5 x 10^4 cells/ml. The following day the medium was replaced by 2.7 mls cold PBSi, placed into the minifreeze unit (in triplicate) and allowed to equilibrate at a particular temperature. Incubations with H_2O_2 under similar conditions were also carried out at 37°C in an incubator and at 4°C in the refrigerator.

4.2.3.2 Modification of H_2O_2 cytotoxicity by various agents

Incubation of cells with glucose (BDH Chemicals) (as a possible source of reducing equivalents), enzyme inhibitors: 3-amino-1,2,4-triazole (Sigma), penicillamine (Sigma) and iron complexes (suppliers and preparations given in section 2.2) were studied to examine the effect of these on the cytotoxicity of H_2O_2.

4.3 RESULTS

4.3.1 Half-life of hydrogen peroxide in the presence of cells

The potassium iodide (KI) assay described in section 2.2.6.1 proved to be a rapid and accurate method for estimating the stock H_2O_2 before each experiment and to measure the half-life of H_2O_2 in the presence of cells. The same results were obtained when H_2O_2 was made up in PBSi at pH 7.40, provided the measurements were made immediately after making up the solution.

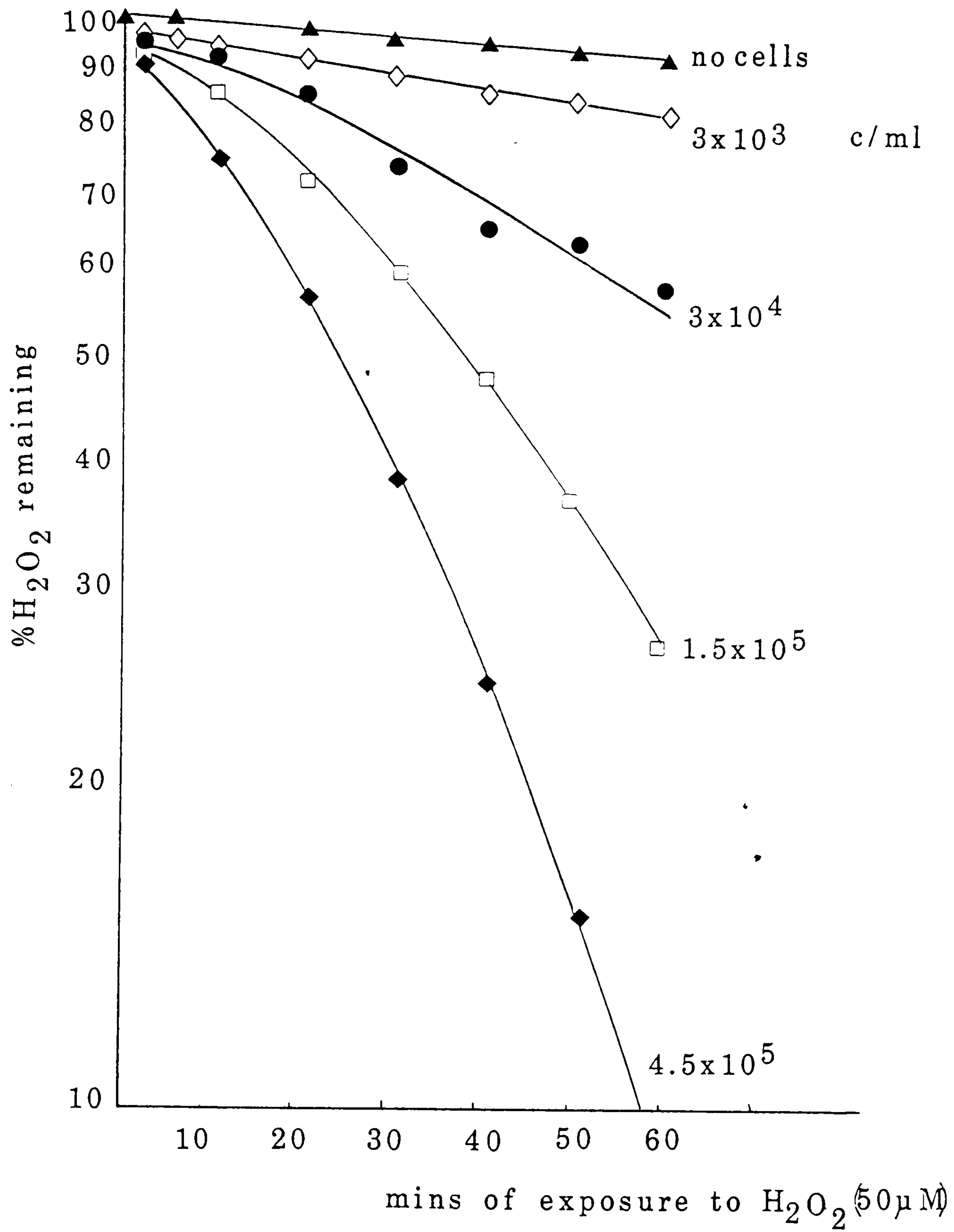
It was found that when cells were exposed at different cell density to H_2O_2 at a constant concentration the survival as assessed either by colony formation (P.E.) or delayed ^{3}H-TdR-incorporation, varied according to the number of cells present at the time of incubation. That this may at least be partly the consequence of degradation of H_2O_2 was demonstrated by exposing cells at different densities to an initial concentration of 50uM H_2O_2.

The rate of decomposition of H_2O_2 was studied in the presence of cells. Cells were seeded at different densities in large flasks. The following day the medium was removed, the cells washed twice with PBS A+B and incubated with PBSi containing 50μM H_2O_2. The stock H_2O_2 solution was measured before each experiment. Flasks contained 15 mls of PBSi with 50 μM H_2O_2 initially. At time intervals of 1,5,10,20,30,40 and 60 minutes aliquots

of 1 ml of the solution were removed. Although the total amount of H_2O_2 to which the cells were exposed was reduced as a result of removing half the initial volume (7 mls) by the end of the 60 minutes, this was not taken into account in calculating the rate of H_2O_2 decomposition by the cells. At each time interval the H_2O_2 was measured by the KI method. The mean rate of removal of H_2O_2 was calculated for various cell densities. The results were examined by linear regression analysis (see Appendix II). After 60 minutes the cells were washed, incubated in growth medium and counted the following day using a Coulter counter by the method described for subculturing cells (section 3.2.9). This was to establish survival as a result of exposure to H_2O_2 and was carried out as a check for comparison with cytotoxicity data. Untreated controls were included.

Fig 4.2 shows the residual H_2O_2 concentration as a function of time of incubation at 37°C for different densities of cells. At low cell density, ie. 3×10^3 cells/ml or 3×10^4 cells/ml the concentration of H_2O_2 to which the cells are exposed after 60 minutes is still relatively high (30-45 μM, ie. 60-90% of the initial concentration), whereas in the presence of 4.5×10^5 cells/ml all the H_2O_2 has been removed by the end of the 60 minute incubation period. In all cases there is an initial rapid rate of degradation removing between 5-10% of the initial concentration. Thereafter the rate of H_2O_2

DECAY OF H_2O_2 INTHE PRESENCE OF CELLS



PBSi pH7.40

fig 4.2

removal is considerably reduced. Thus, two phases are evident which are concerned with the degradation of H_2O_2; the first which is almost instantaneous and the second which occurs at a steady rate over the remaining 60 minutes.

The control represents a flask containing no cells and shows that under the same conditions there is a small spontaneous decay of H_2O_2. The half-life of H_2O_2 in the presence of various cell densities is summarised in table 4.1.

These results are not inconsistent with the data of Hoffmann et al. (1984) who obtained decomposition rates of H_2O_2 with 3 different cell lines with half-lives of 52 minutes for 5 x 10^5 cells with an initial concentration of H_2O_2 of 100µM in phosphate buffer.

The degradation rate was calculated using the calculation described in Appendix III. The mean rate of removal (k) was estimated from the slope of a plot of log (C_t/C_o) vs t using regression analysis, details of which are given in Appendix II. This was calculated for each cell density and plotted against the estimated number of cells present at the time of the experiment (fig 4.3). Despite the variablity the results suggest that the degradation rate varies linearly with cell number. The estimated total dose of H_2O_2 to which cells are exposed during a 60 minute incubation period may be calculated from the integral of the decay expression which is

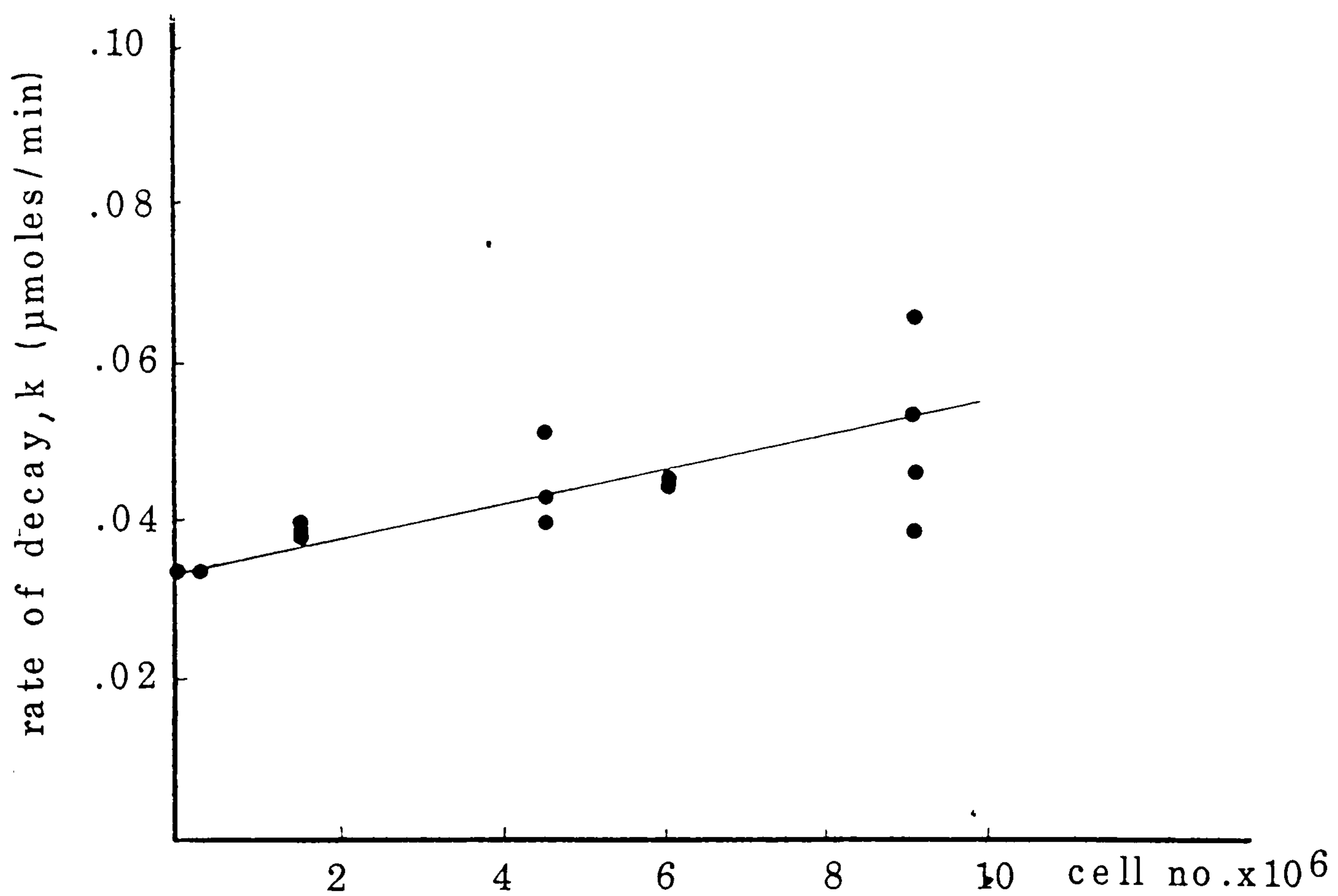
TABLE 4.1

HALF-LIFE OF H_2O_2 AS A FUNCTION OF CELL DENSITY

Cell density (c/ml)	Mean t½ (mins)
3×10^3	>100
3×10^4	77
1.5×10^5	37.5
4.5×10^5	29
6×10^5	13
1.2×10^6	10

c/ml = cells/ml
Total volume in flask at time of seeding: 10mls
Initial concentration of H_2O_2: 50 µM
Medium used in experiment: PBSi (15 mls initially)
Incubation at 37°C, in a CO_2 incubator, pH 7.40

RATE OF H_2O_2 REMOVAL WITH VARYING CELL NUMBER



regression data:

T= 4.19

2P= 0.001

R= 0.75

intercept=.0338

slope = 2.31×10^{-9}

fig 4.3

described in Appendix IV together with a short programme.

Fig 4.3 illustrates the straight line equation $k = aN + C$. From the regression analysis the intercept (C) is 0.0338 with a slope of 2.31×10^{-9} N, where N equals the number of cells. By substitution for k into the equation for the total dose, D was calculated for different cell numbers used. This calculation was used in an attempt to 'normalise' the data obtained under varying conditions of H_2O_2 concentration to take into account any changes due to the variation in cell number. The relationship between the total dose of H_2O_2 and the number of cells which are exposed for 60 minutes to an initial concentration of 50 μM H_2O_2 is shown in fig 4.4 and illustrates that the dose during every minute of exposure to H_2O_2 is linearly related to the cell number. Comparitive survival was estimated by cell numbers after 24 hours (table 4.2) which shows strong dependency on cell density. The effects observed were confirmed by cytotoxicity data (see section 4.3.2).

The following sections describe the effects of addition of various agents which are reported to affect the metabolism of H_2O_2.

4.3.1.1 Addition of glucose

The half-life of hydrogen peroxide was measured in the presence of 1 mM glucose in order to test whether, by providing sufficient reducing equivalents for the glutathione peroxidase (GPx) catabolism of H_2O_2, the rate

TOTAL DOSE OF H_2O_2 AS A FUCTION OF CELL NUMBER

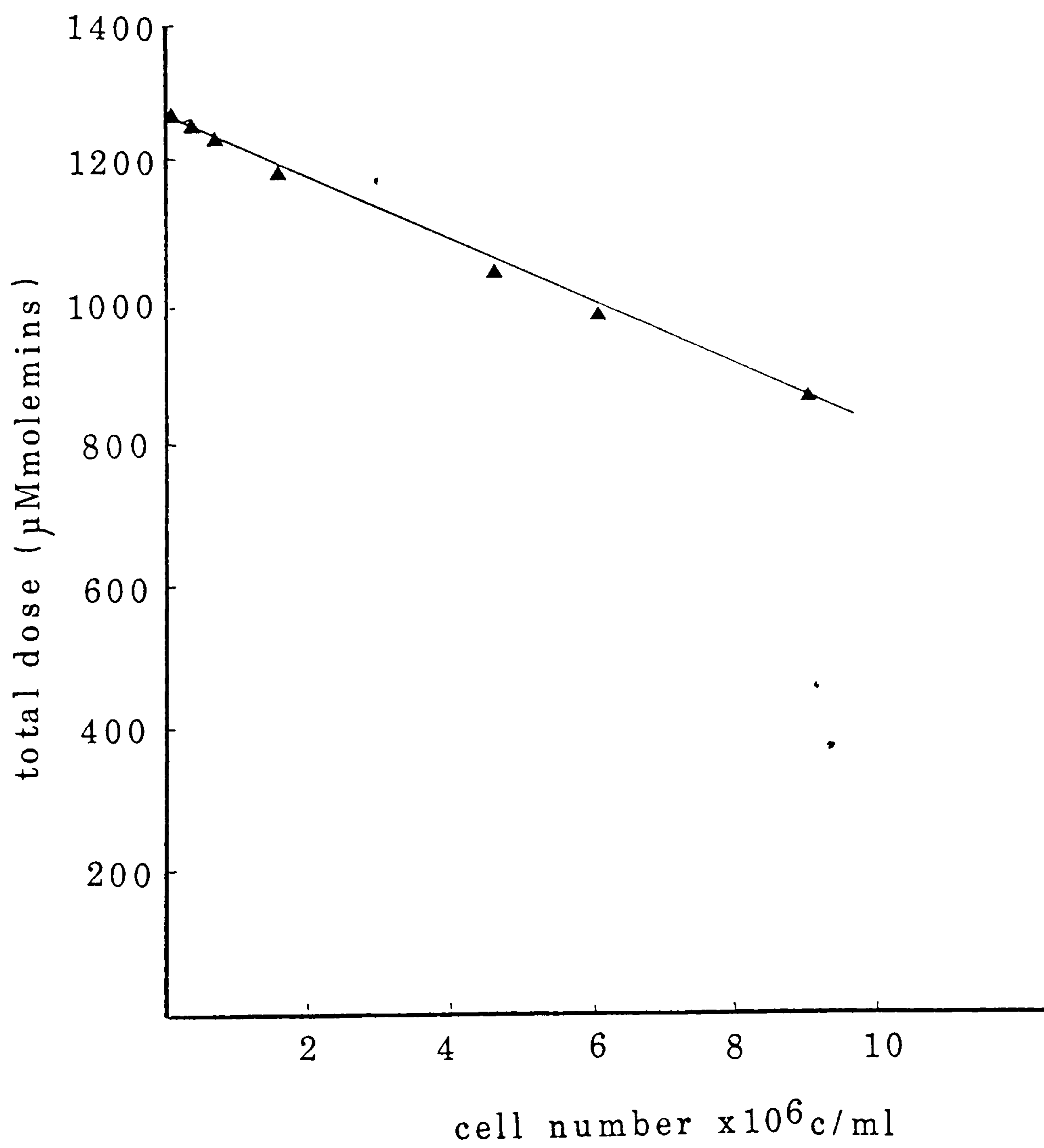


fig 4.4

TABLE 4.2

RECOVERY OF CELLS 24 HOURS AFTER TREATMENT WITH H_2O_2

seeding density c/ml	control no treatment counts c/ml	control + 50μM H_2O_2 counts c/ml	% recovery
5×10^5	2×10^5	9.7×10^4	49
1.5×10^5	3.1×10^5	2.7×10^5	92

of degradation could be affected.

Cells were exposed to 50 µM H_2O_2 in PBSi at 37°C in the presence of 1mM glucose which was added to the cells 10 minutes after the beginning of the exposure period. 100 µl of a 300 mM stock solution was added to the flask to minimise the effect of dilution. Cells were washed, placed in growth medium at 37°C and counted using a Coulter counter after 24 hours.

The addition of glucose after 10 minutes of incubation with H_2O_2 decreased the half-life of H_2O_2 only slightly; t1/2 = 17 minutes compared to t1/2 = 20 minutes in the absence of glucose (fig 4.5). These cells were at a density of 1-2 x 10^5 cells/ ml at the time of the experiment. However, on counting the cells 24 hours later it was evident that the cells were completely protected from damage by H_2O_2 when treated with 1 mM glucose under these conditions (table 4.3) despite the fact that the glucose was not initially present. This result was confirmed by the cytotoxicity data (section 4.3.2.5) and suggests that protection by glucose is not related to the degradation of H_2O_2 by the cells.

4.3.1.2 Effects of 3-amino-1,2,4-triazole (3-ATZ)

Cells were pre-incubated with 10 mM 3-ATZ, a known inhibitor of catalase (Stark & Farber, 1985), for 2 hours in medium. The cells were then washed and re-incubated with 50 µM H_2O_2 in PBSi at 37°C for 60 minutes. Measurements of residual H_2O_2 were made on aliquots as

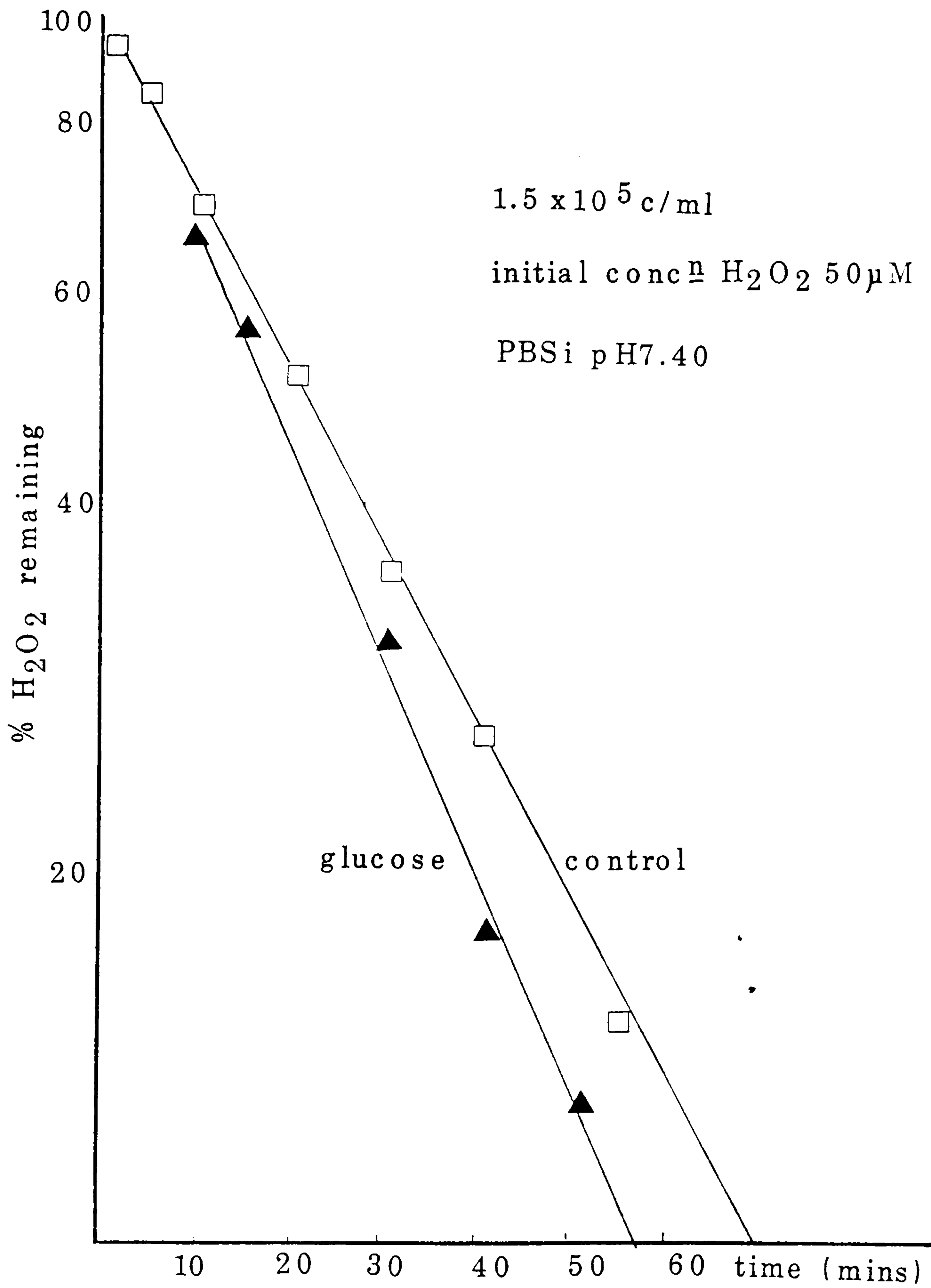


fig 4.5

TABLE 4.3

EFFECT OF D-GLUCOSE ON RECOVERY OF CELLS 24 HOURS AFTER EXPOSURE TO H_2O_2

Additions	cell count after 24 hours (c/ml)	% recovery
-	6×10^6	100
50 μM H_2O_2	2.9×10^6	48
50 μM H_2O_2 +1 mM D-glucose after 10 mins	6.3×10^6	105

before. Cells were washed, placed in growth medium at 37°C and counted 24 hours later.

The initial rate of degradation of H_2O_2 was not affected in the first 10 minutes of measurement but was partially inhibited thereafter (fig 4.6). The mean half-life of H_2O_2 in the presence of 3-ATZ treated cells was 45 minutes, compared to $t½$ = 20 minutes for control cells. However, this difference was not reflected by an increased cytotoxicity of cells measured 24 hours after treatment, (table 4.4). The inhibition of the degradation of H_2O_2 10 minutes after exposure thus has no effect on the subsequent viability of the cells. This was confirmed by cytotoxicity data in section 4.3.2.6

4.3.1.3 Effects of penicillamine (PCA)

Penicillamine (β-mercaptoethanol), a known inhibitor of glutathione peroxidase (Chaudierre *et al*.,1984) was dissolved in distilled water. Cells were pre-incubated with 10 mM PCA in medium for 2 hours. This was followed by washing in PBS A+B and incubation with 50 μM H_2O_2 in PBSi. This experiment was carried out in multiwells. Each set of 4 wells contained cells which were exposed to H_2O_2 for different time intervals when the PBSi was removed and the H_2O_2 measured. 1 ml growth medium was added per well and the survival index (S.I.) measured 24 hours later by the ^{3}H-TdR incorporation assay (as described in 3.2.12.3).

An increased rate of removal of H_2O_2 was observed

EFFECT OF PRE-INCUBATION OF CELLS WITH 3-ATZ ON H_2O_2 DEGRADATION

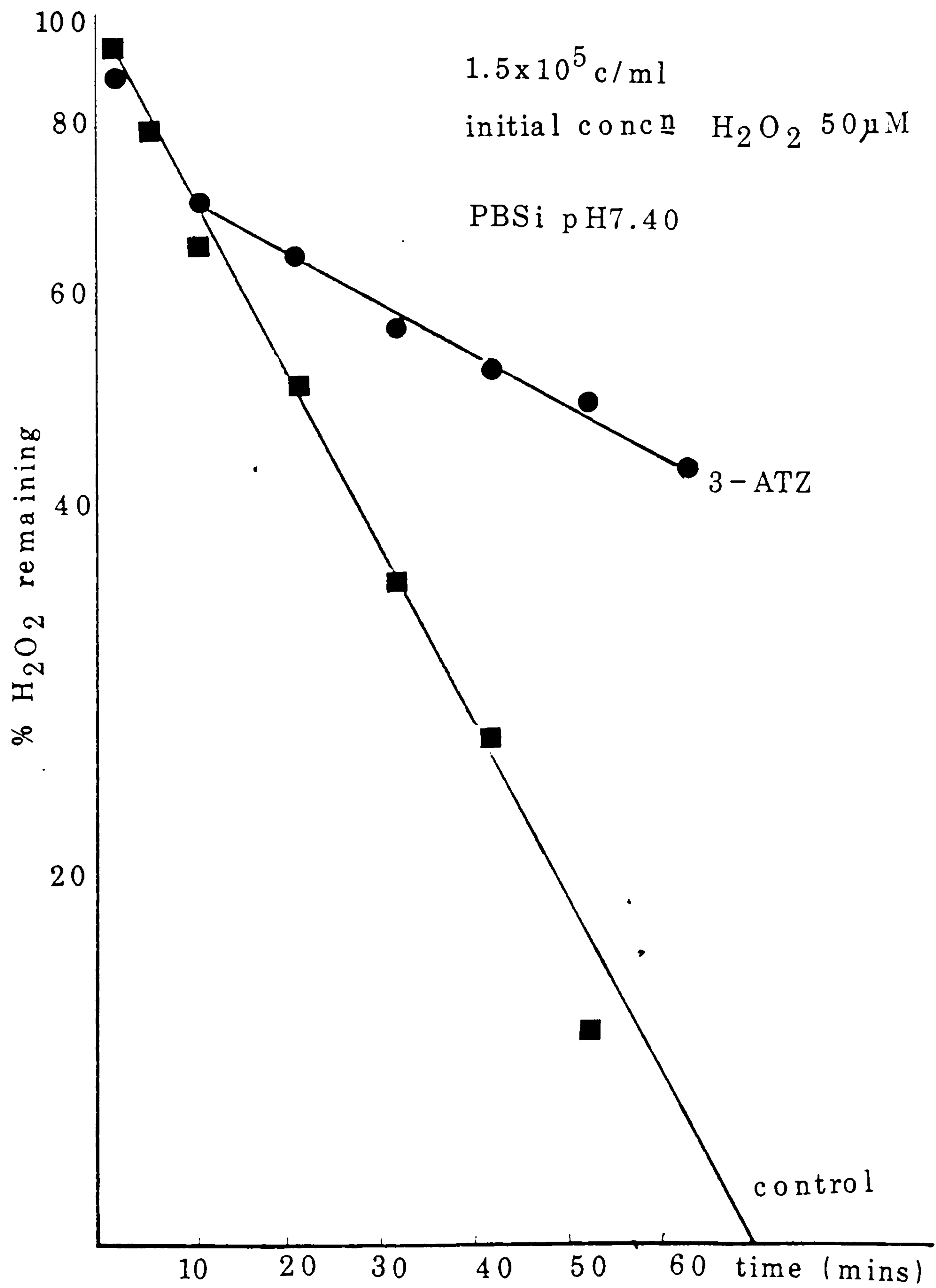


fig 4.6

TABLE 4.4

EFFECT OF PRE-TREATMENT WITH 3-ATZ ON RECOVERY OF CELLS 24 HOURS AFTER EXPOSURE TO H_2O_2

Additions	cell counts after 24 hours c/ml	% recovery
control	6×10^6	100
50 μM H_2O_2	3.6×10^6	63
pre-incubation with 3-ATZ 2 hrs + 50 μM H_2O_2	4×10^6	67

on pre-incubation with 10 mM penicillamine for 2 hours. The effect was biphasic. Almost 50% H_2O_2 was removed by PCA pre-treated cells within the first minute of exposure to H_2O_2, thereafter the rate of degradation had a half-life of 27 mins (fig 4.7), which is comparable to the half life of 25 mins in the absence of PCA following an initial drop of nearly 20% under these conditions. The experiment was made in multiwell dishes in which the cells were estimated to be at a density of 10^5 cells/ml.

4.3.1.4 Effect of pH

GPx is also reported to be sensitive to pH (Chaudierre, 1984). To examine whether the degradation rate could be affected by altering the pH cells were incubated either in multiwells or in large flasks with PBSi adjusted to pH between 6 and 7 and exposed to 50 µM H_2O_2 for 60 minutes.

Fig 4.8 shows that by lowering the pH the rate of removal of H_2O_2 from the medium decreases. The half-lives at pH 7.4 and pH 6.41 were 19.5 and 26.5 minutes respectively. Cells were used at a density of 1.5×10^5 cells /ml.

4.3.1.5 Effect of boiled cells

To test whether the increased rate of H_2O_2 degradation in the presence of cells was dependent on enzymatic activity cells seeded at a density of 4.5×10^5 cells/ml were heated to 100°C for 10 minutes and fixed in 4% glutaraldehyde for a further 10 minutes. After cooling

EFFECT OF PCA ON THE DEGRADATION OF H_2O_2 BY CELLS

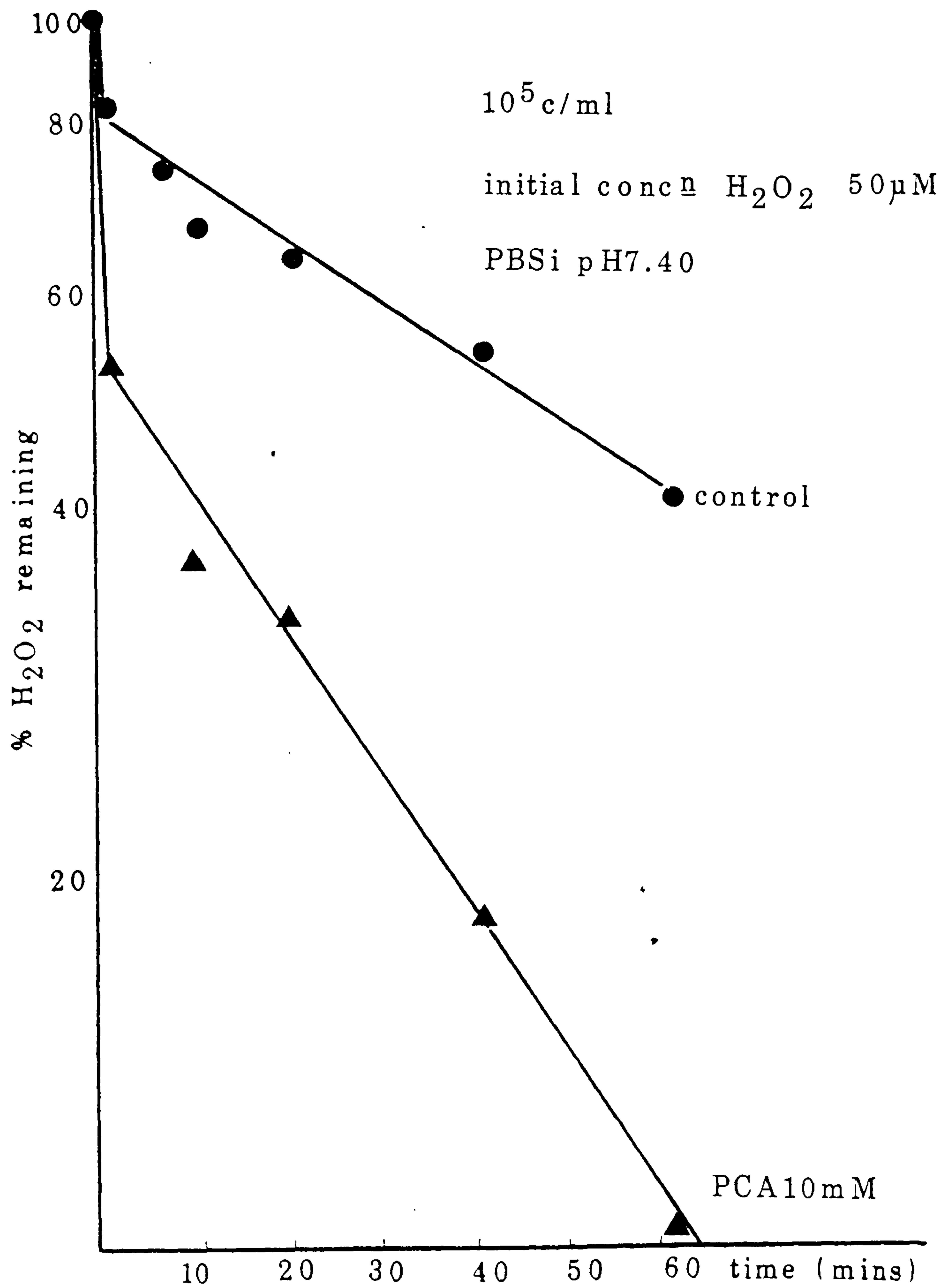


fig 4.7

EFFECT OF pH ON THE RATE OF H_2O_2 DEGRADATION BY CELLS

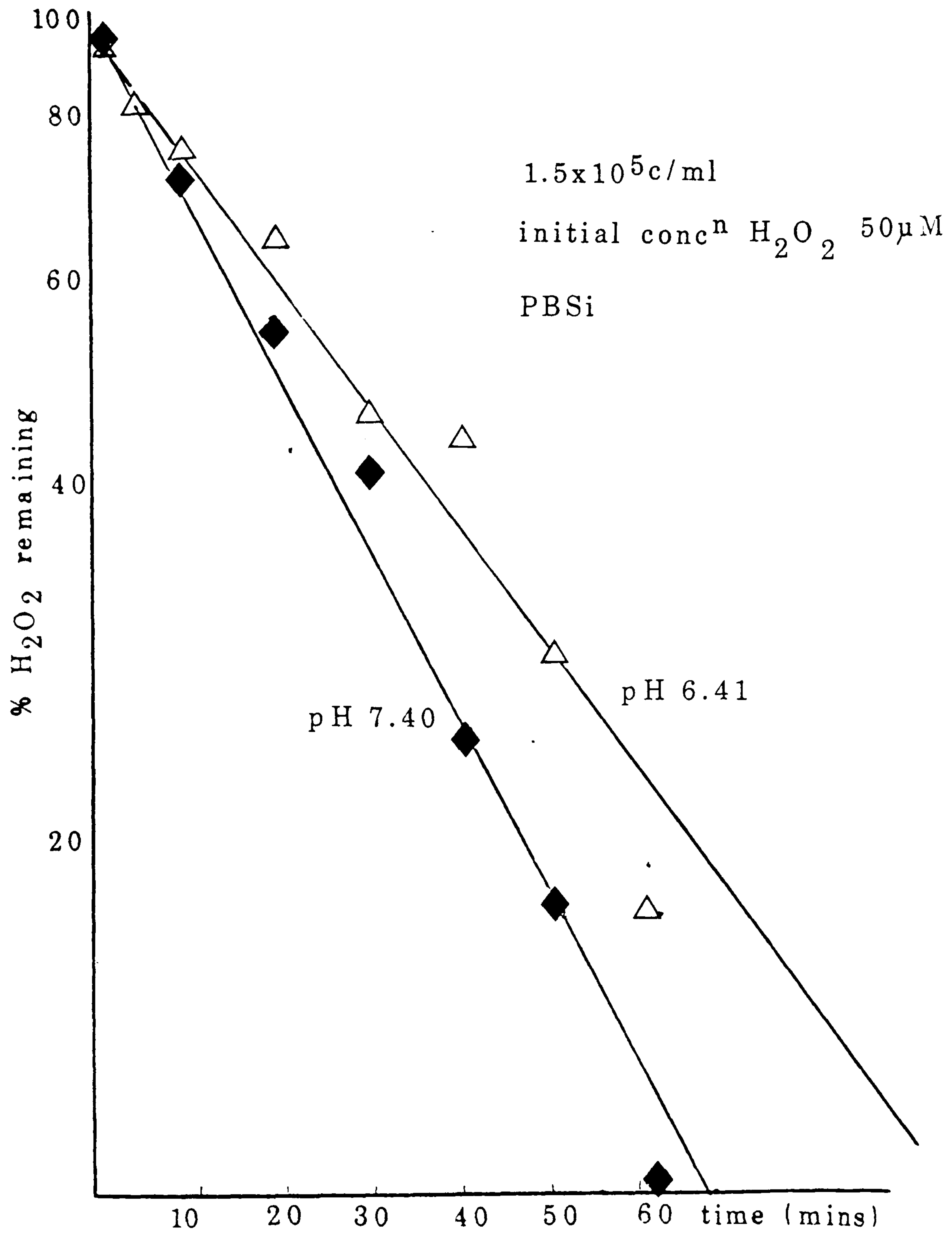


fig 4.8

the H_2O_2 solution was added to the flask and aliquots taken for H_2O_2 estimation at intervals.

Fig 4.9 shows that H_2O_2 degradation is inhibited.

4.3.1.6 Effect of temperature

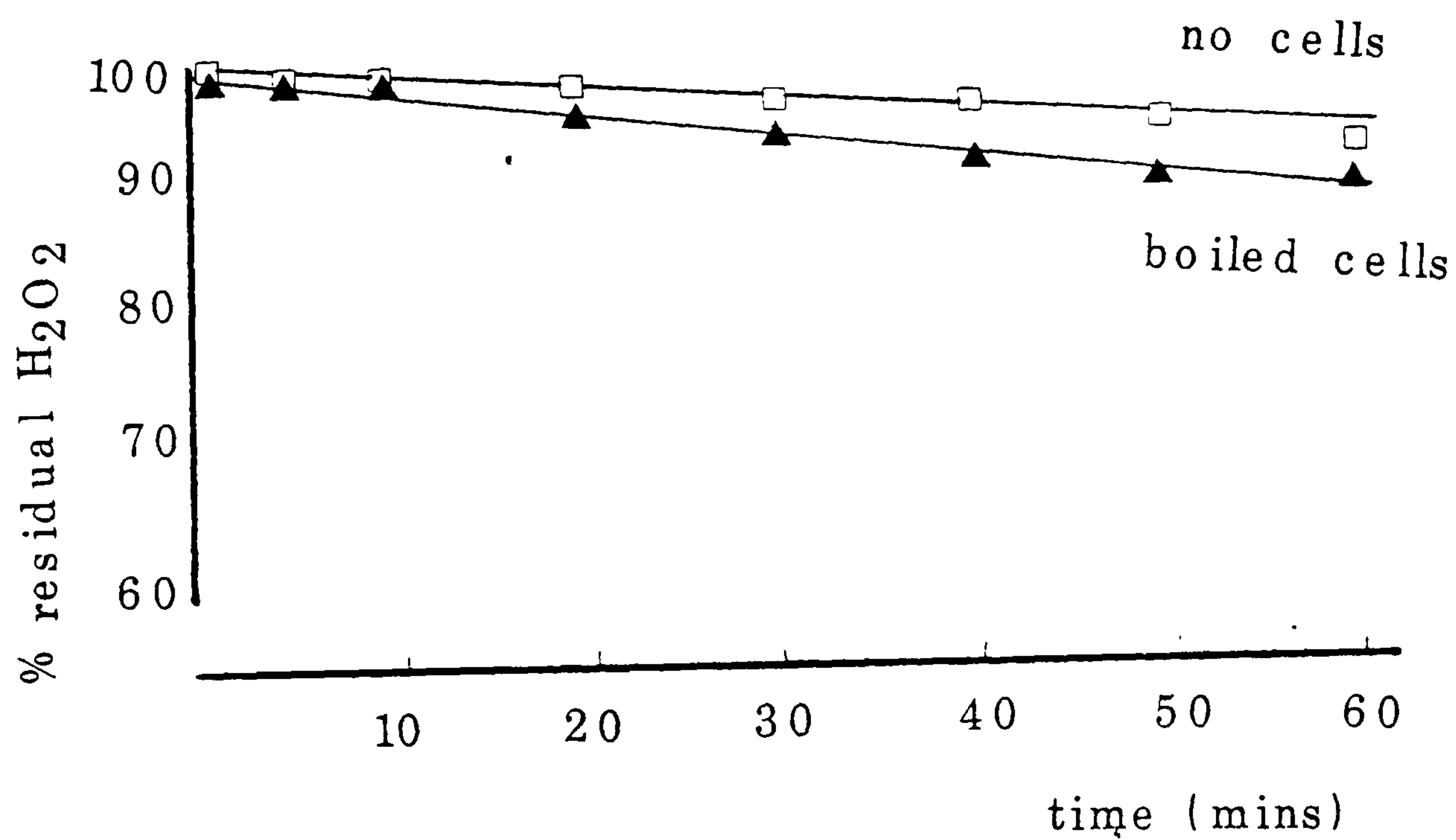
Possible temperature dependent metabolic processes involving the removal of H_2O_2 were examined by measurement of residual H_2O_2 from cell incubations at 4°C and 37°C. This was carried out in multiwell trays in which cells, at a density of 10^5 cells/ml, were exposed to PBSi containing 50µM H_2O_2 for 60 minutes.

Fig 4.10 demonstrates that the rate of degradation of H_2O_2 by the cells in the second phase is not significantly affected by a change in temperature. It is known that the activity of catalase is virtually unaffected by changes in temperature down to 0°C (Komov & Schmelev,1976). At 4°C the rate of removal of H_2O_2 was 0.16 µM/min compared to 0.21 µM/min at 37°C. Thus at 4°C cells are exposed to a slightly higher concentration of H_2O_2 during the incubation period. However, the initial phase of rapid removal of H_2O_2 (ie 10% in 1 min) at 37°C is inhibited at 4°C suggesting that only this phase is affected by these temperatures.

4.3.2 Cytotoxicity assays

Several studies were carried out to investigate the effect of H_2O_2 on cell survival and how this effect can be manipulated by the addition of various different agents.

EFFECT OF BOILED CELLS ON THE RATE OF H_2O_2 REMOVAL



4.5×10^5 c/ml
PBSi pH7.40
initial conc^n H_2O_2 50µM

fig 4.9

RATE OF H_2O_2 REMOVAL BY CELLS AT 37°C AND 4°C

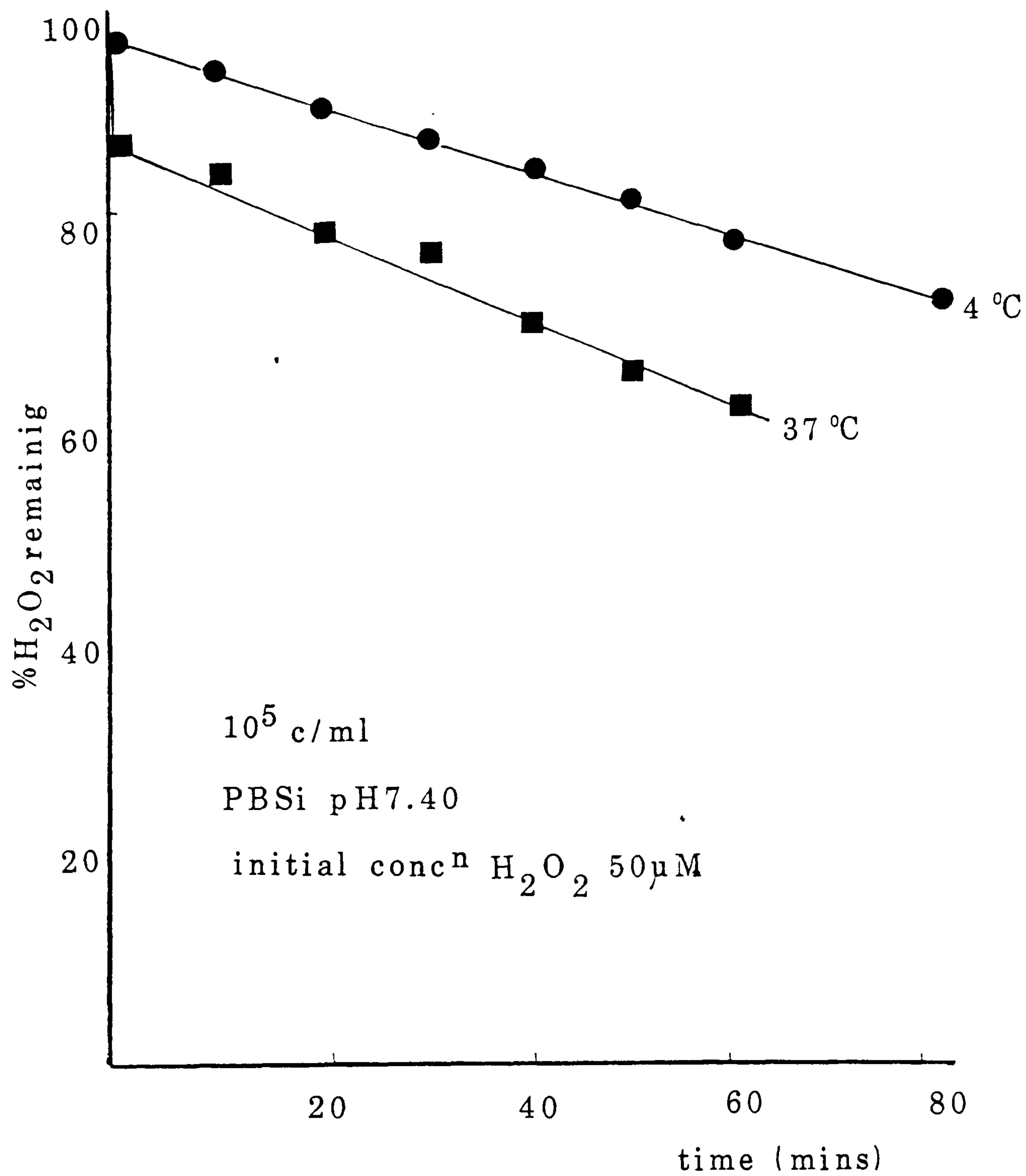


fig 4.10

4.3.2.1 Plating efficiency

Cells at a final concentration of 5 x 10^4 cells/ml were exposed to increasing doses of H_2O_2 for 60 minutes in suspension in PBSi at 37°C. Immediately following this cells were diluted 1 in 10 with medium, counted in a haemocytometer chamber and further diluted to give 200 cells per petri dish. Plating efficiency was determined as described in section 3.2.11.

The surviving fraction of cells exposed for 60 minutes to increasing concentrations of H_2O_2 is illustrated in fig 4.11. The near-linear plot of log surviving fraction vs dose implies that at this cell density the relationship of survival against dose follows the general equation of $S = S_o.e^{-D}$ (Coggle,1983), where D=dose, S = surviving fraction, and S_o is the surviving fraction of untreated cells.

4.3.2.2 ^{3}H-TdR-incorporation

Thymidine incorporation assays were carried out on cells 24 hours after exposure. The procedure is described in section 3.2.12. Cells were exposed to H_2O_2 in PBSi at increasing concentrations for 60 minutes after which they were washed and re-incubated in medium for 24 hours prior to the ^{3}H-TdR incorporation assay.

4.3.2.2.1 Passage number

A variable which was noted was the increase in the rate of cell proliferation during successive passages which may in some cases have accounted for variation in

SURVIVAL OF CNCM 221 CELLS EXPOSED TO H_2O_2

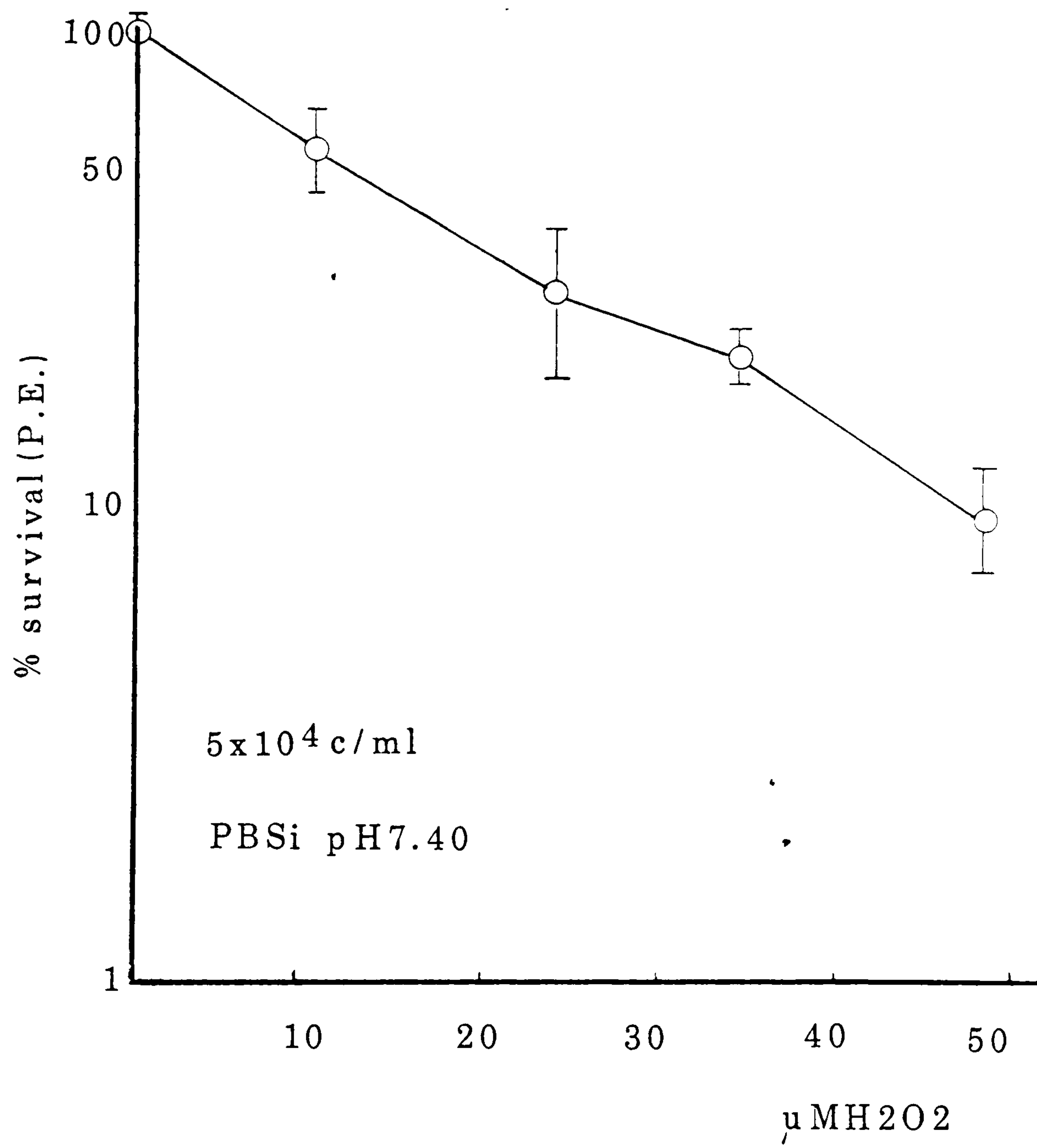


fig 4.11

experiments. This was reflected in higher levels of ^3H-TdR incorporation in cells seeded at the same density. Table 4.5 gives the ^3H-TdR incorporation data as counts per minute for control cultures of CNCM I-221 cells from several experiments.

4.3.2.2.2 Survival Index

Cells seeded in multiwells at different densities were exposed to H_2O_2 for 1 hour at 37°C in PBSi. Fig 4.12 shows the survival as estimated by ^3H-TdR incorporation 24 hours after treatment. If ^3H-TdR incorporation is carried out immediately after treatment with H_2O_2 no difference between treated and untreated cells is seen. When labelling was carried out 24 hours after exposure of 3×10^5 cells/ml to 50 μM H_2O_2 a 56 % reduction in labelling compared to controls was evident. The lack of an immediate effect implies that DNA synthesis is not impaired in exposed cells. The subsequent reduction in labelling is regarded as an indicator of the proportion of viable cells remaining that are capable of DNA replication. The comparison with the controls is distorted by the proliferation of the control cultures during the intervening period. For example, in CNCM I-221 cells approximately 3 doublings would be expected to occur in 48 hours; thus the 48-hour survival index will represent a significant underestimate of the actual survival. For this reason the 24-hour post incubation time was employed and the fraction of cells incorporating

TABLE 4.5

VARIATION OF THYMIDINE INCORPORATION WITH PASSAGE NUMBER OF CNCM I 221 CELLS

passage number	mean cpm	S.D
25	34838	2201
26	39092	15856
27	42222	4434
28	61426	11109
29	88821	5726
30	105423	16101
33	114383	12944

^{3}H-TdR after this delay is referred to as the survival index (S.I.). In fig 4.12 the data represent the mean of quadruple estimates. At high cell densities a characteristic 'shoulder' is observed at low H_2O_2 concentration; an effect which has been noted with H_2O_2 by others (Link & Riley,1988; Hoffmann _et al_.,1984) and which is similar to the effect of irradiation of cells (Alper,1987; Elkind _et al_.,1987).

4.3.2.3 Comparison of the plating efficiency with survival index

The correlation between the results from the plating efficiency assay and 24-hour post-exposure ^{3}H-TdR incorporation was investigated by experiments carried out at similar cell densities. ^{3}H-TdR incorporation data obtained at 2 different passages were compared with plating efficiency data. In fig 4.13 survival is plotted as percentages of the relevant controls in both cases. The sigmoidal curves indicate that at low H_2O_2 concentration the ^{3}H-TdR incorporation assay is apparently more sensitive than plating efficiency. This suggests that a proportion of cells which are unable to incorporate the label after 24 hours are nevertheless able to recover and form colonies. The cells at lower passage number show a greater sensitivity to treatment with H_2O_2, which may reflect the comparitive cell densities at the time of exposure.

In view of the more rapid and adequate method of

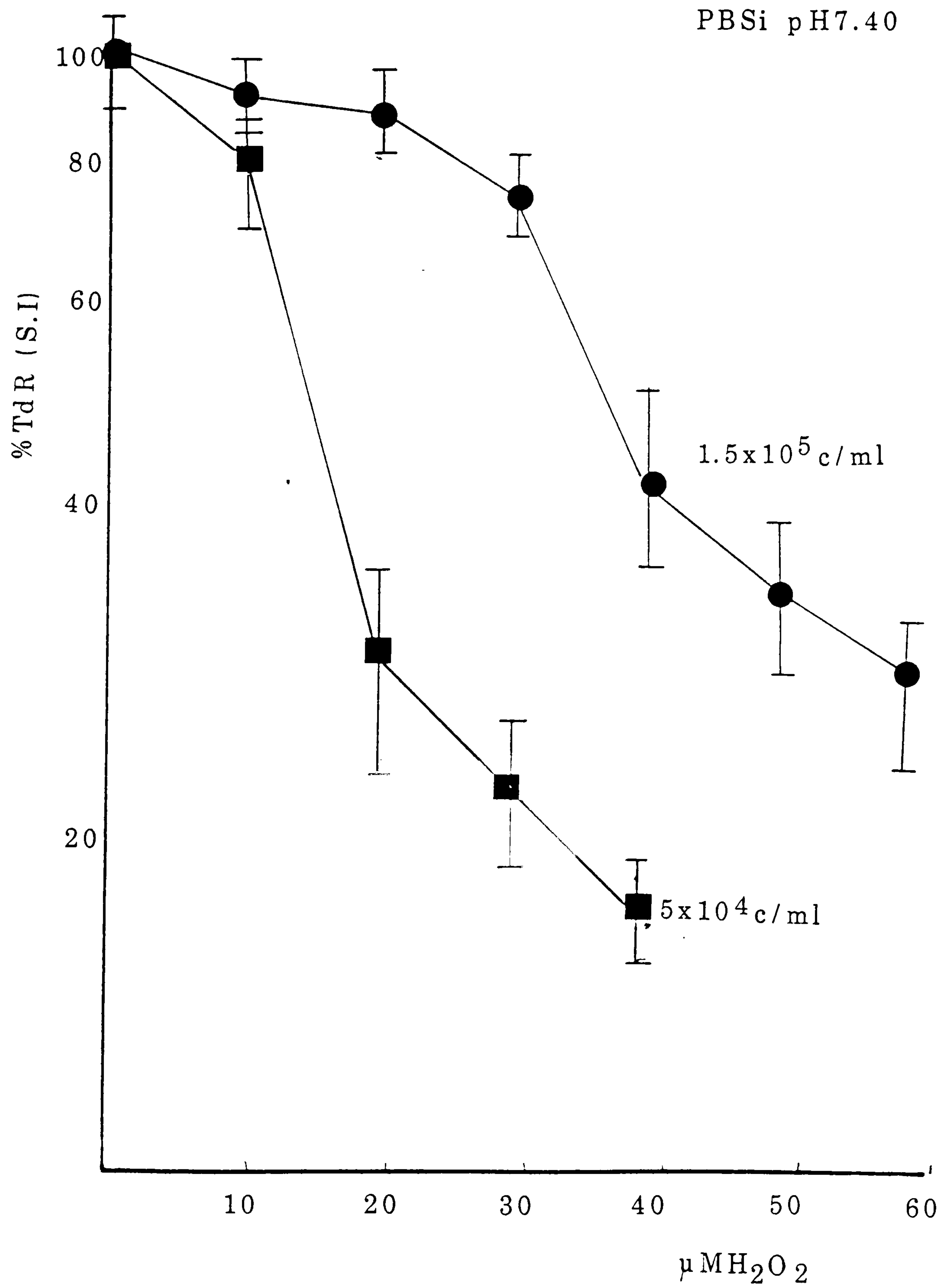
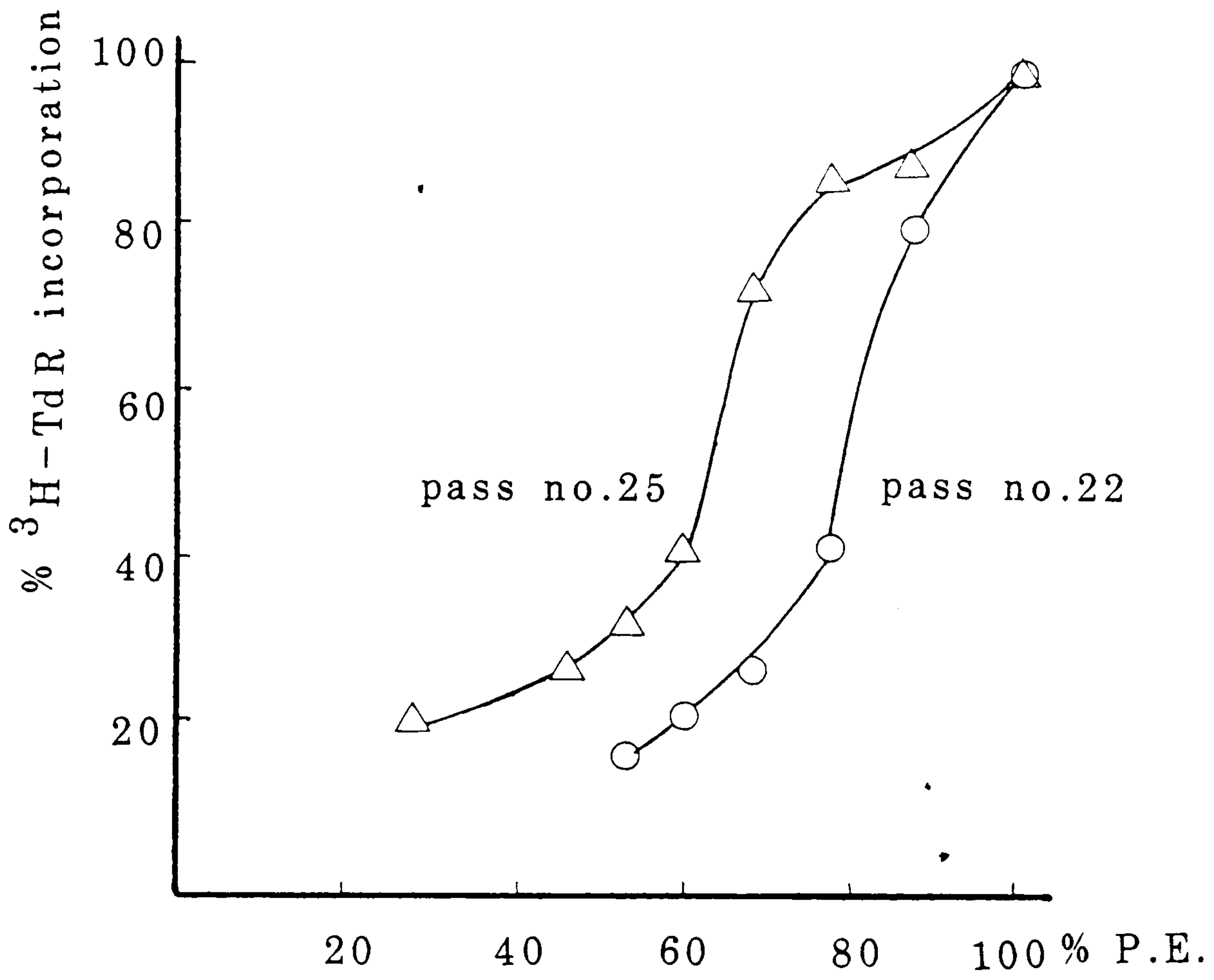


fig4 12

CORRELATION OF ^{3}H-TdR INCORPORATION WITH PLATING EFFICIENCY



10^{5} c/ml

fig 4.13

estimating the survival of cells it was opted to use ^{3}H-TdR incorporation as a suitable assay.

4.3.2.4 Effect of exposure to H_2O_2 on cell viability studied by the survival index (S.I.)

Using the ^{3}H-TdR-incorporation assay the effect of H_2O_2 on cell survival was investigated under different conditions.

Fig 4.14 summarizes the effect of the number of cells on the S.I. for different doses of H_2O_2. The data are plotted as the mean survival against cell number at each concentration of H_2O_2. Saturation of the response is evident at every tested concentration of H_2O_2. The critical cell density after which the slope of survival versus cell number changes is approximately 1.5 x 10^5 cells/ml in all cases. This may be an artifact due to the limitation of the multiwell surface area. Beyond this critical density the cells approach confluency and the growth rate is impeded. This diminution of growth rate with increasing cell density is well documented and several suggestions have been made with regard to the factors which govern growth regulation (Stoker & Rubin, 1967; Loewenstein,1968; Houck et al.,1972).

From the calculations of total dose in section 4.3.1 the data have been presented (fig 4.15) as the S.I. versus total dose, which takes the relative rates of degradation into account. The data have been compiled from several different experiments involving varying

Legend to fig 4.14:

CNCM-221 cells were seeded at different densities and exposed to H_2O_2 at different concentrations in PBSi pH 7.40.

% S.I AS A FUNCTION OF CELL NO.
AT VARIOUS CONCENTRATIONS OF H_2O_2

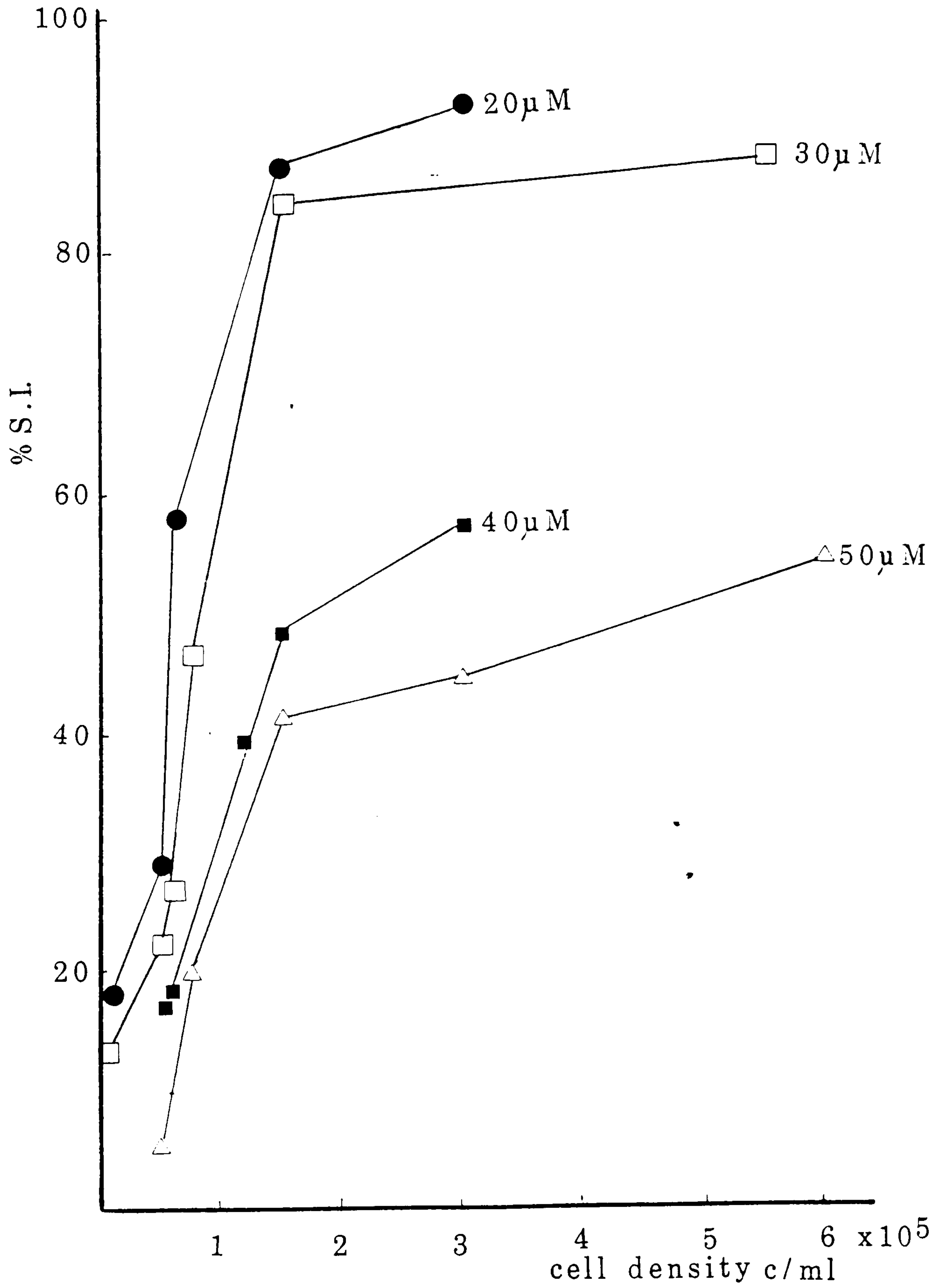


fig 4.14

Legend to fig 4.15:

Regression data:
regression of Y on X
intercept at 81.49%
T = -3.65
2P = 0.001 with 30 degrees of freedom
correlation coefficient = -0.56
slope = -0.04X

REGRESSION PLOT FOR % S.I. AS A FUNCTION OF TOTAL DOSE OF H_2O_2

(showing no correlation between total dose and % S.I.)

% S.I.

100
80
60
40
20

200 400 600 800 1000 1200 1400 1600 1800 2000

total dose (μmolmins)

fig 4.15

numbers of cells and the line obtained from linear regression analysis. Since the correlation coefficient was -0.56, there appears to be no correlation between individual points of total dose and the survival. However, the highly significant probability supports the relationship of decreasing survival with increasing dose.

Using the data presented in fig 4.14 the dependency on cell density can be eliminated by expressing the dose in terms of moles of H_2O_2 per cell (fig 4.16). In this case a good correlation exists between survival and dose/cell. The dependency of H_2O_2-induced cytotoxicity on the cell density has been noted previously (Ziegler-Skylakis & Andrea,1987; Spitz et al.,1987). Elimination of this dependency was also achieved by plotting the data in terms of μmoles/cell.

The following experiments were performed to examine the effect of agents and varying conditions on the viability of CNCM I-221 cells, some of which were shown previously to affect the rate of degradation of H_2O_2 and which have been shown by others to affect cellular response to H_2O_2.

4.3.2.5 Effect of addition of glucose

Cells were grown in multiwells. The medium was removed and replaced by PBSi containing glucose 1mM and H_2O_2 and the cells incubated at 37°C for 1 hour prior to processing for the delayed ^{3}H-TdR incorporation assay.

Addition of glucose to the H_2O_2 solution protected

Legend to fig 4.16:

Regression data:
correlation coefficient = -0.91
2P < 0.001 with 15 degrees of freedom
slope = 0.01X
intercept at 95%

REGRESSION LINE FOR S.I. WITH INCREASING DOSE OF H_2O_2

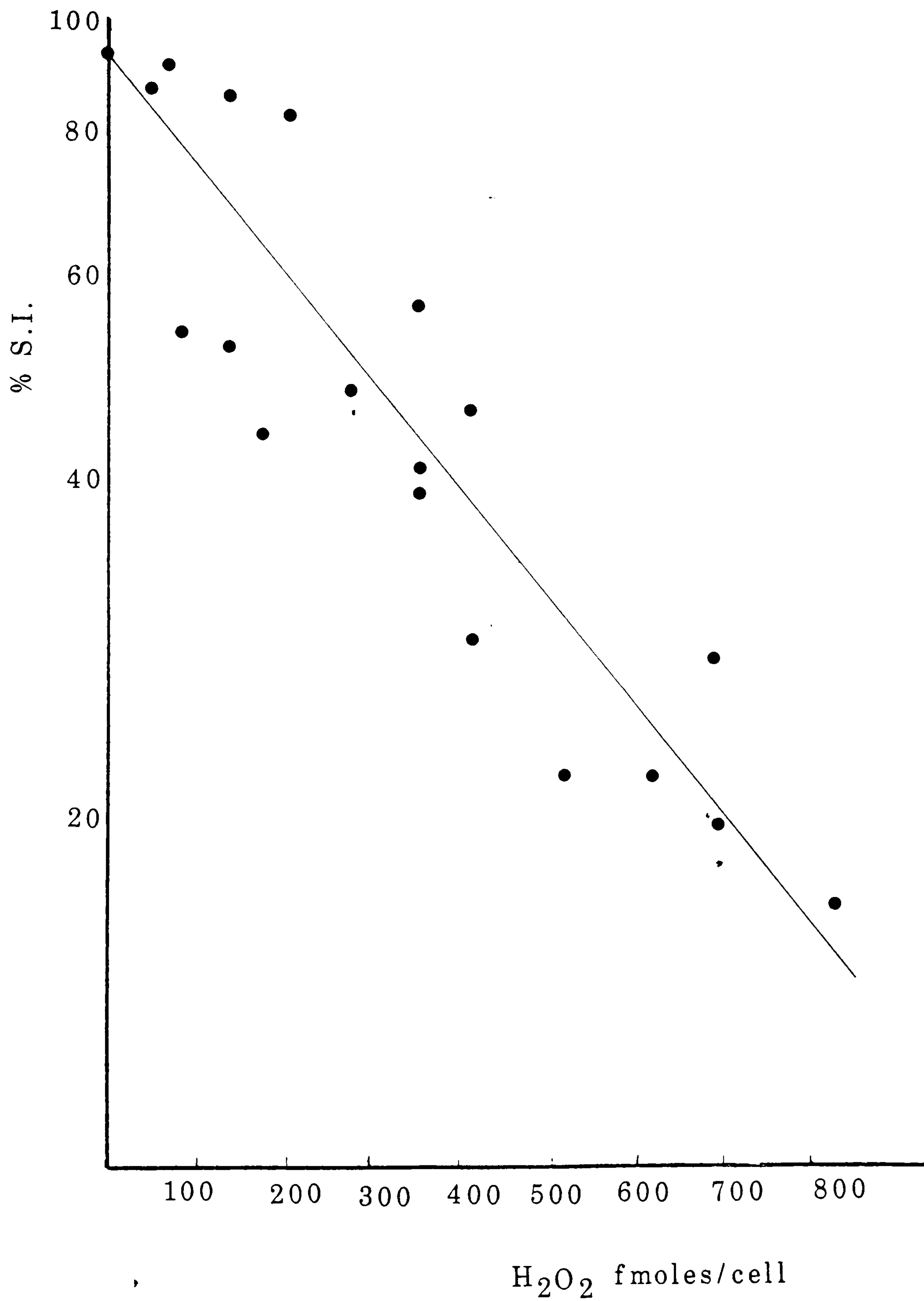


fig 4.16

the cells (table 4.6). The rate of removal of H_2O_2 in the presence of glucose was measured previously (section 4.3.1.1). The half-life of H_2O_2 in the presence or absence of glucose was not significantly different. This suggests, that glucose protects cells by a mechanism other than by affecting the removal rate of H_2O_2. Since glucose was previously shown to protect cells even it was added 10 minutes after exposure to H_2O_2 (section 4.3.1.1), the protection must be occurring in the second phase of the incubation period.

4.3.2.6 Effect of pre-incubation with 3-ATZ

3-ATZ at a final concentration of 20 mM was added to attached cells in serum-containing medium for 2 hours. The medium was removed, the cells washed and replaced by PBSi containing 50 μM H_2O_2. The cells were incubated with H_2O_2 for various time intervals between 0 and 60 minutes at 37°C. Following washing and incubation in growth medium at 37°C the delayed ^{3}H-TdR incorporation assay was performed as described previously.

A concentration of 20 mM was used by Stark & Farber (1985) who also used a prolonged pre-incubation period. Fig 4.17 shows that 3-ATZ had no significant effect on the toxicity of H_2O_2. The rate removal of H_2O_2 is inhibited by pre-exposure of cells to 3-ATZ by about 50% after the first 10 minutes (fig 4.6).The cells are thus exposed to a higher concentration of H_2O_2 by the end of the incubation period. The rate of H_2O_2 degradation was

TABLE 4.6 EFFECT OF D-GLUCOSE ON CELLS EXPOSED TO H_2O_2

Glucose conc^n (mM)	H_2O_2 (50μM)	% S.I. ± S.D.
0	0	100 ± 10
0	+	41 ± 4
0.8	0	83 ± 6
0.8	+	62 ± 13
1.0	+	73 ± 0

Significance brackets in the S.I. column: 100 ± 10 vs 41 ± 4 (a); 41 ± 4 vs 62 ± 13 (b); 83 ± 6 vs 62 ± 13 (b); 41 ± 4 vs 73 ± 0 (a)

(a) $p < 0.005$ (b) $p < 0.05$

glucose and H_2O_2 added simultaneously

Legend to fig 4.17:

CNCM-221 cells pre-incubated with 3-ATZ for 2 hrs in SC MEM.

EFFECT OF 3-ATZ ON THE S.I OF CELLS

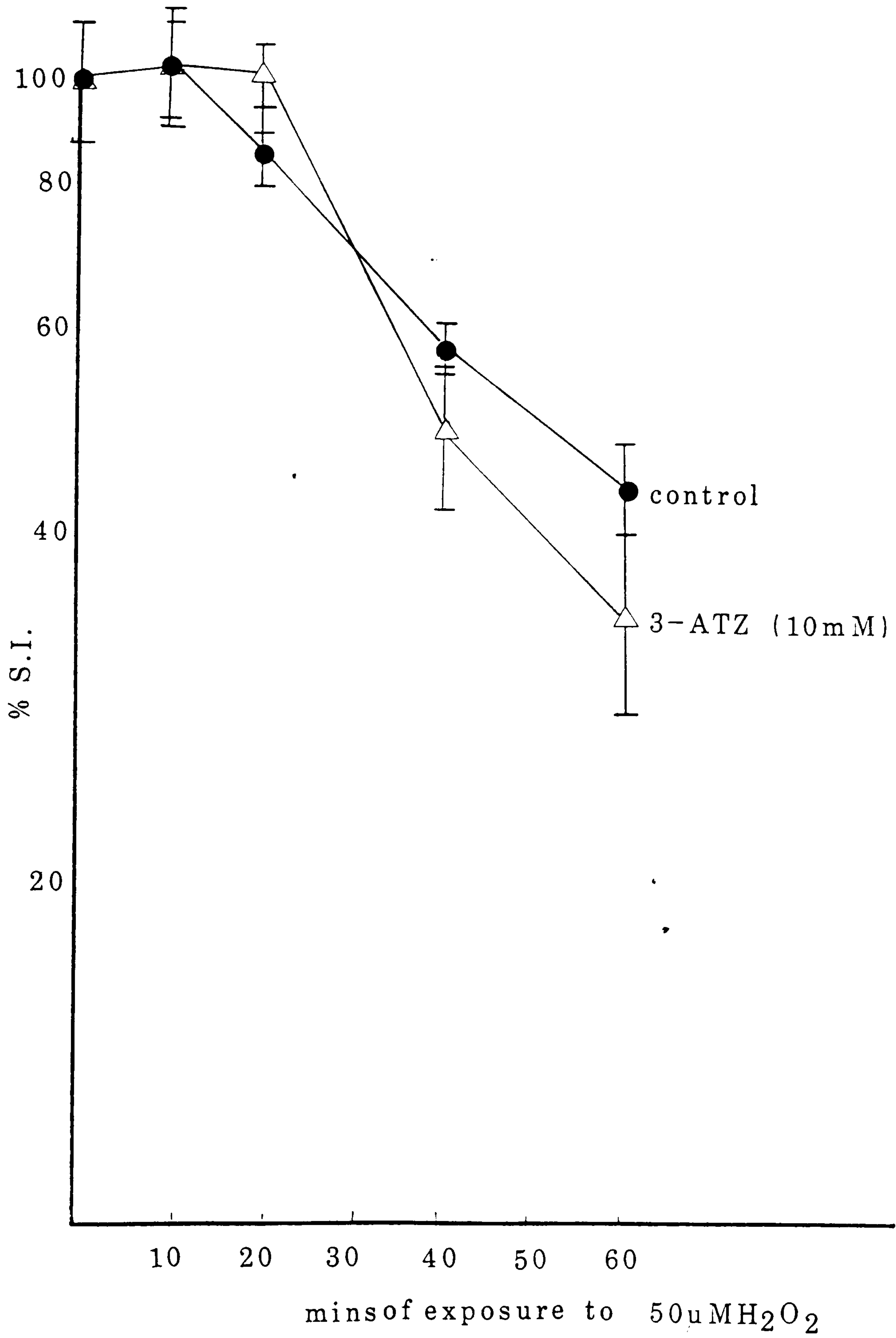


fig 4.17

not affected during the first 10 minutes. It appears that under these conditions inhibition of catalase does not affect the toxic action of H_2O_2. The failure to observe an increase in cytotoxicity contrasts with results by Stark & Farber (1985), who found that cell killing was potentiated in rat liver hepatocytes which were pre-treated with 3-ATZ and then exposed to H_2O_2 generated either by glucose oxidase or by the metabolism of menadione. However, therein are cited experiments showing that not all cells respond to 3-ATZ in this way (Nathan et al.,1981; Arrick et al.,1982).

4.3.2.7 Effect of penicillamine (PCA)

Cells were exposed to 10 mM PCA for 2 hours in serum-containing medium, then washed and incubated with 50 µM H_2O_2 for different times in PBSi at 37°C prior to washing, incubation in growth medium at 37°C and ^3H-TdR incorporation after 24 hours.

Pre-incubation with 10 mM PCA shows a significant increase in the cytotoxic action of H_2O_2 (fig 4.18). Under the same conditions the initial rate of removal of H_2O_2 is significantly increased in the presence of PCA (fig 4.7).

Since PCA only exaggerated the first phase of the biphasic pattern of H_2O_2 degradation the toxic effect may be related to this initial phase.

4.3.2.8 Effect of pH

Cells were incubated in PBSi which was previously

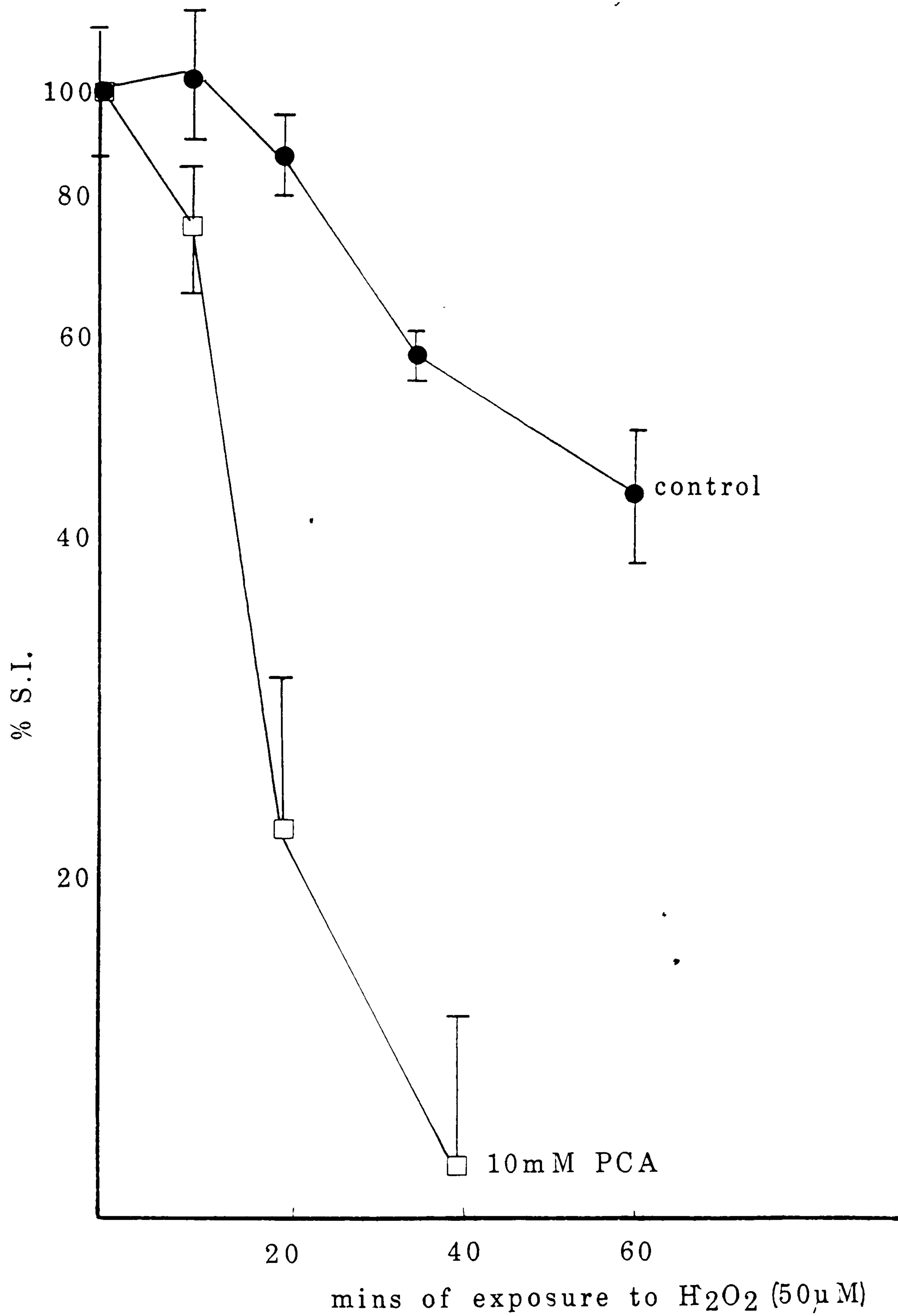


fig 4.18

adjusted to pH 6.5 by addition of 1M NaOH. Incubation with 50 μM H_2O_2 was carried out for different time intervals. This was followed by processing for the delayed ^{3}H-TdR incorporation assay.

Lowering the pH to 6.5 enhances the toxicity of H_2O_2 (fig 4.19). The rate of removal of H_2O_2 (fig 4.8) decreases as the pH is lowered. This has the effect of exposing cells to a higher concentration of H_2O_2 during the incubation period and is consistent with the increased cytotoxicity observed. The sensitivity of GPx to pH has been shown by others (Chaudierre et al.,1984), who have demonstrated the pH optimum for this enzyme to be 8.5. Lowering the pH from 7.4 to 6.5 would be expected to diminish the activity of GPx, consistent with the observations. However, the reduced rate of degradation of H_2O_2 is reflected in a greater increase in cytotoxicity than would be expected from the calculation of total dose of H_2O_2 to which the cells are exposed at pH 6.4 (c.f. fig 4.8 and 4.19). Changing the extracellular pH may not necessarily result in intracellular changes in pH. A lower pH may result in decreased spontaneous H_2O_2 degradation.

4.3.2.9 Effects of temperature on the survival index

Cells seeded at 5 x 10^4 cells/ml in multiwell trays the previous day were washed with PBS A+B at either 37°C or 4°C and incubated in PBSi at these temperatures for a few minutes. 50 μM H_2O_2 was added to these and the cells

EFFECT OF ALTERING pH ON THE S.I. OF CELLS

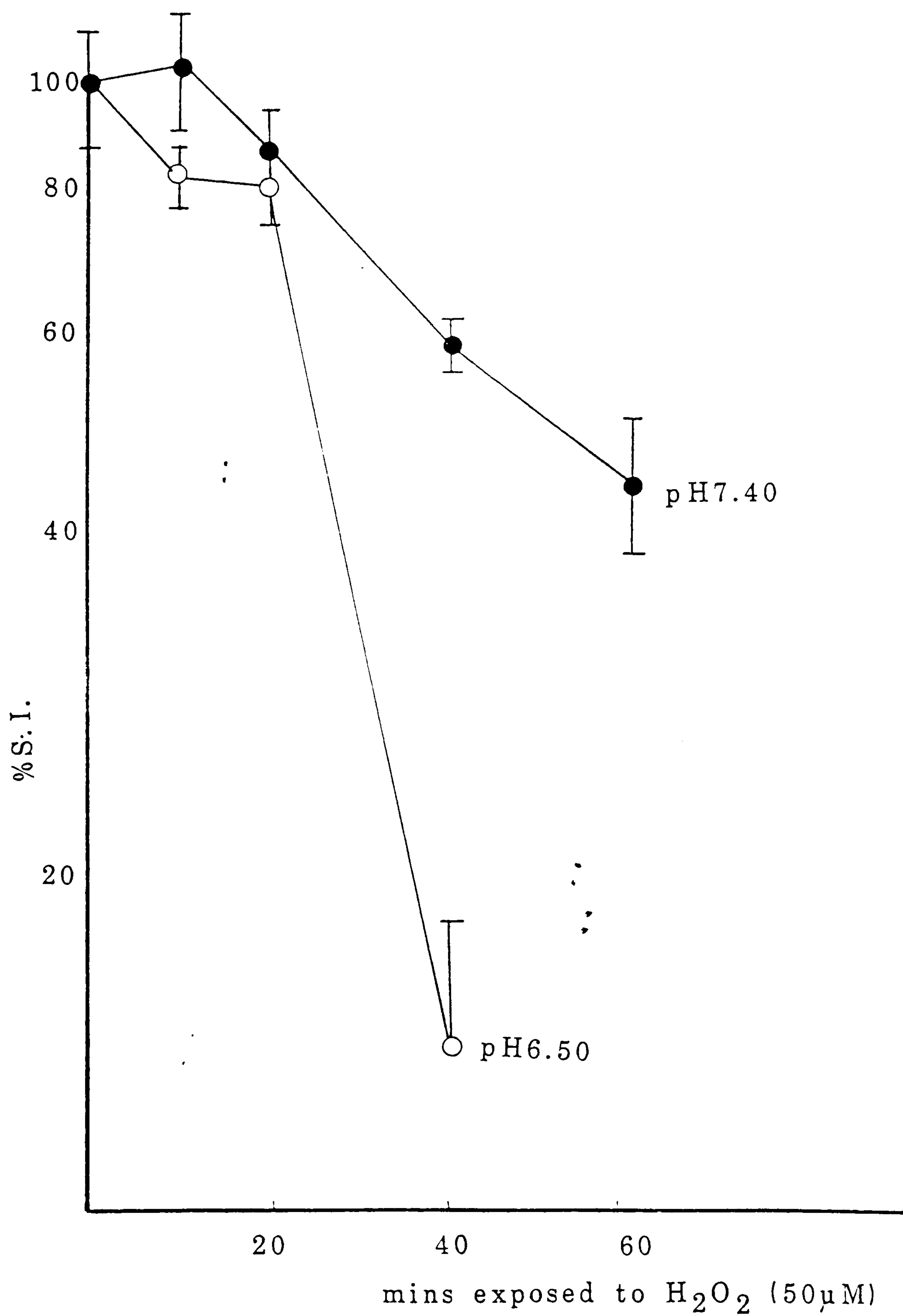


fig 4.19

incubated at 37°C in an incubator or at 4°C in a refrigerator for 60 minutes. H_2O_2 was replaced by medium at 37°C and ^3H-TdR incorporation assessed after 24 hours. Results from several experiments with cells exposed to H_2O_2 at 37°C and at 4°C were compared using linear regression analysis as described in Appendix II. The results from several experiments were plotted as S.I. against moles of H_2O_2 per cell (fig 4.20). The discrepancy from the regression in fig 4.16 is possibly due to differences in passage numbers between the two different sets of experiments. The data presented in fig 4.16 come from later experiments, ie higher passage numbers. Cells exposed to H_2O_2 at 4°C exhibited no dose-dependent reduction in survival ($r= 0.42$) whereas cells exposed at 37°C showed an exponential loss of viablity ($r= 0.98$). This effect of low temperature in abrogating cell killing by H_2O_2 has previously been observed by Ward et al. (1985), who have shown that at 0°C very high concentrations of H_2O_2 (5×10^{-2}M), are required to produce significant cytotoxicity.

In a further study the effect of a range of temperatures between 4°C and 37°C was investigated. The procedure for this was described in section 4.2. Cells at an approximate density of 1.5×10^5 cells/ml were exposed to 100 μM of H_2O_2 for 60 minutes. The data illustrated in fig 4.21, show a steep gradient between 22°C and 27°C.

The following experiments were carried out in an

Legend to fig 4.20:

Regression data:

4°C: slope = 0
intercept = $\log_{10} 1.98$
correlation coefficient = 0.42

37°C: slope = $-1.75\times10^{-3}X$
intercept at $\log_{10} 1.99$
correlation coefficient = -0.98
$P < 0.001$

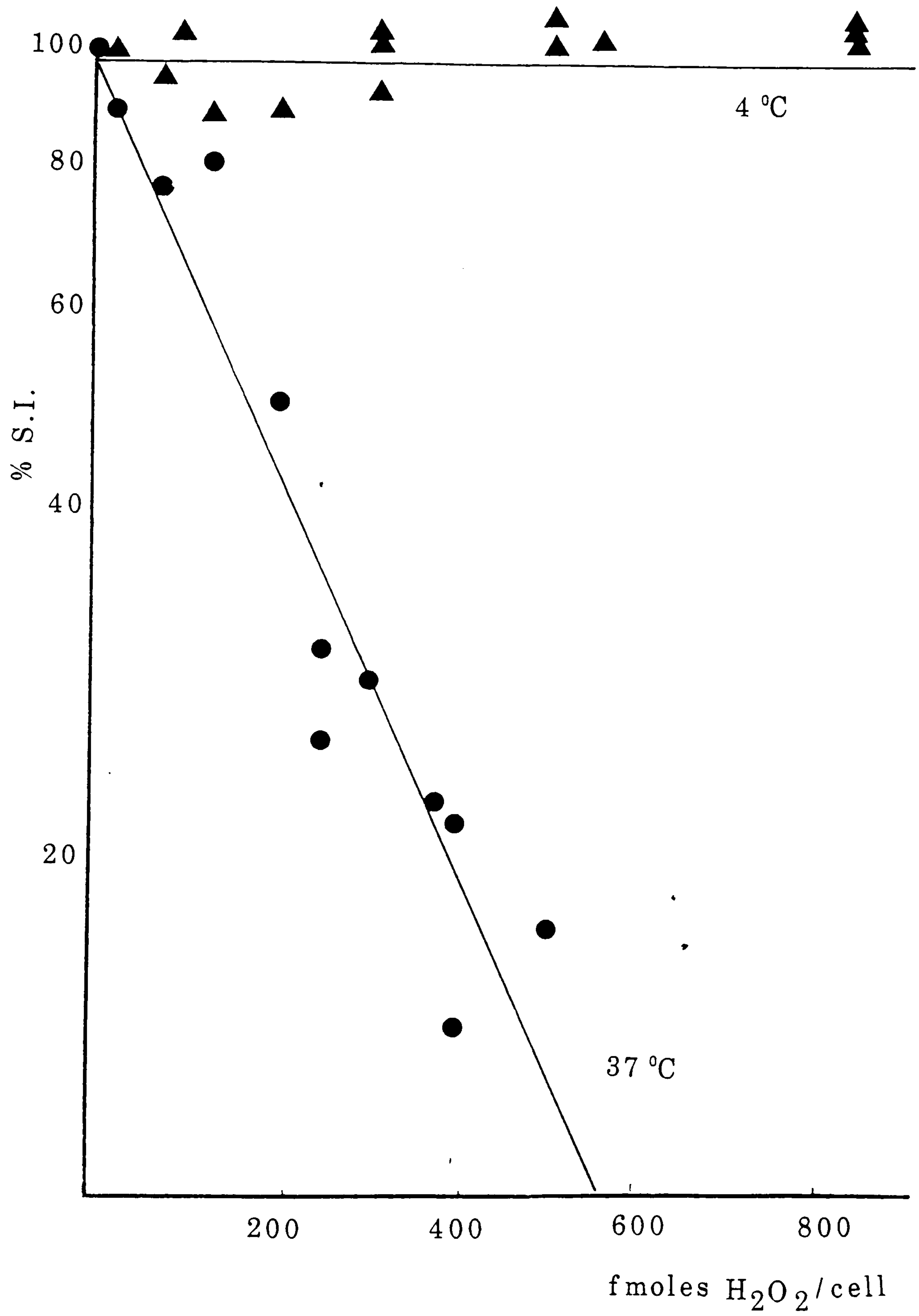


fig 4.20

EFFECT OF TEMPERATURE ON THE S.I OF 221-CNCM CELLS IN THE PRESENCE OF H_2O_2

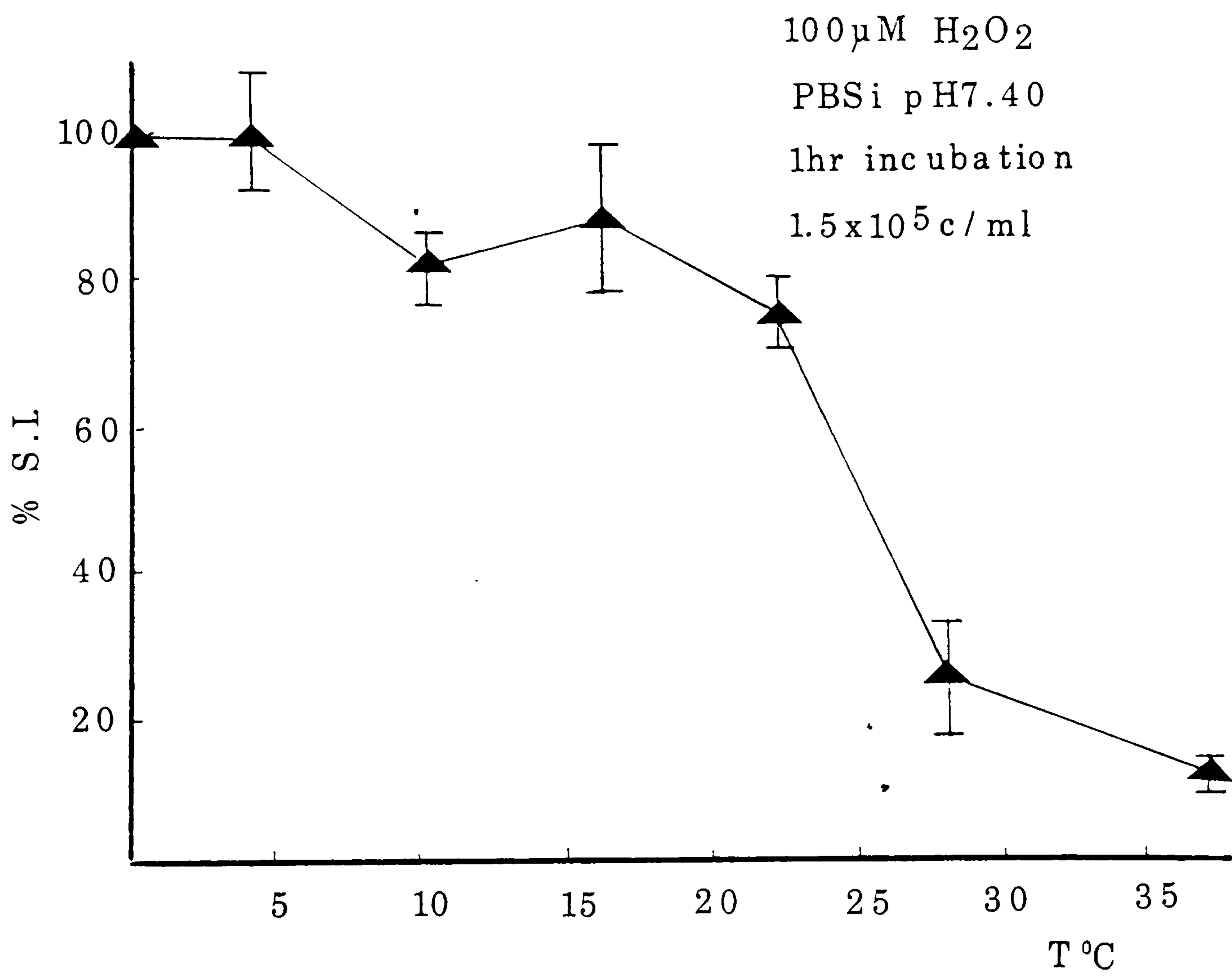


fig 4.21

attempt to restore the cytotoxicity of H_2O_2 at 4°C by the providing reducing agents to the cell.

4.3.2.9.1 Effect of Ascorbic acid and dehydroascorbate pre-incubation

18-24 hours after seeding at a density of 5×10^4 cells/ml in multiwell dishes cells were preincubated with 1 mM ascorbic acid for 2 hours or 5 mM ascorbic acid in medium at either 4°C or 37°C for 1 hour. The cells were then washed with cold PBS A+B and exposed to 50 µM H_2O_2 in PBSi at 4°C for 60 minutes, after which the cells were washed again and incubated in medium for 24 hours followed by ^{3}H-TdR incorporation. Similar experiments were carried out with pre-incubation of cells with dehydroascorbate at different concentrations.

Cytotoxicity of H_2O_2 in the concentration range that is effective at 37°C was observed at 4°C in cells that had been pre-incubated with ascorbate (table 4.7). A prolonged pre-incubation period was required as periods of exposure to 1mM ascorbate for less than 2 hours had little or no effect. Analogous experiments using dehydroascorbate (DHA) demonstrated that the effect was dependent on the temperature of the pre-incubations (table 4.8). DHA pre-incubation at 4°C exhibited a toxic action independent of the addition of H_2O_2, but at 37°C pre-incubation with DHA had the effect of restoring the toxic action of H_2O_2 at 4°C in a manner similar to that seen with ascorbate.

TABLE 4.7

RESTORATION OF THE CYTOTOXICITY OF H_2O_2 AT 4°C BY PRE-INCUBATION WITH L-ASCORBATE

Preincubation* 120' Agent	T°C	Incubation H_2O_2 (50μM) 60'	% S.I. ± S.D. 37°C	4°C
-	37	0	100 ± 14	100 ± 9
			} (a)	} n.s.
-	"	+	46 ± 6	91 ± 17
			n.s	(a)
Ascorbate 1mM	"	0	107 ± 20	99 ± 14
			} (a)	} (a)
"	"	+	39 ± 8	43 ± 4
-	4	0	100 ± 18	100 ± 18
			} (a)	} n.s.
-	"	+	42 ± 16	90 ± 15
Ascorbate 1mM	"	0	-	108 ± 17
				} n.s.
"	"	+	-	89 ± 21

(a) $P<0.005$; n.s. $P>0.05$

* Cells at ~1.5 x 10^5 c/ml were incubated in serum-containing medium (SC MEM) containing ascorbate. After washing with PBS A+B, cells were incubated with PBSi containing H_2O_2.

TABLE 4.8

RESTORATION OF THE CYTOTOXICITY OF H_2O_2 AT 4°C BY DEHYDROASCORBATE (DHA)

Preincubation[1] 120' Agent	T°C	Incubation H_2O_2 (50μM) 60'	% S.I. ± S.D. 4°C
-	37	0	100 ± 9 } n.s.
-	"	+	91 ± 17
DHA 50μM	"	0	96 ± 5 } n.s.
"	"	+	94 ± 30 — [2] n.s.
" 100μM	"	0	95 ± 20 } (b)
"	"	+	62 ± 11 — (b)
" 1 mM	"	0	90 ± 11 } (a)
"	"	+	46 ± 12 — (a)
-	4	0	100 ± 18 } n.s.
-	"	+	90 ± 15
DHA 1 mM	"	0	55 ± 13 } n.s.
"	"	+	69 ± 20

(a) $P<0.005$; (b) $P<0.05$; n.s. $P>0.05$

[1] Details of incubations given in legend to table 5.8

[2] compared to cells treated with H_2O_2 alone.

4.3.2.9.2 Effects of lipoic acid (LPA)

Alternative reducing agents were also tested. Reduced and oxidised lipoic acid were dissolved in 50% ethanol. This limited the concentration of lipoic acid to which cells could be exposed; the final concentration was such that the maximum amount of ethanol was at a non-toxic level of 1%.

Cells were exposed to 100, 200 or 500μM lipoic acid at either 4°C or 37°C, washed and incubated with PBSi containing 50μM H_2O_2 for 60 minutes prior to the delayed ^3H-TdR incorporation assay.

Pre-incubation with reduced LPA also re-established H_2O_2-induced cytotoxicity at 4°C. The solubility of LPA limited the maximum concentration that could be used. Oxidised LPA was ineffective in restoring cytotoxicity. Despite the greater lipophilicity of LPA, pre-incubation at 4°C with reduced LPA did not restore the cytotoxic action of H_2O_2 (table 4.9) even at the higher concentration used. At 37°C 500μM LPA is toxic by itself.

4.3.2.9.3 Protection by desferrioxamine (DFO)

Cells were seeded in multiwells at 2 x 10^4 cells/ml. When attached, DFO at various concentrations (50-200μM) was added to the medium. Incubation was carried out for 24 hours. The cells were then washed with PBS A+B and incubated with 1 mM ascorbic acid for 2 hours at 37°C followed by washing in cold PBS A+B and incubation at 4°C with H_2O_2. The protocol is summarized

TABLE 4.9

EFFECT OF PRE-INCUBATION WITH LIPOIC ACID ON THE ABSENCE OF CYTOTOXICITY AT 4°C BY H_2O_2

Pre-incubation *Agent 120' (µM)	[4]T°C	Exposure to H_2O_2 60' 4°C (50µM)	% S.I. ± S.D.
-	37	0	100 ± 6 } n.s.
-		+	103 ± 19
RLPA[1] (100)		0	113 ± 19 } (a)
		+	71 ± 4 (a)[2]
(200)		0	N.A.
		+	62 ± 12 (a)[2]
OLPA[3] (100)		0	100 ± 10 } n.s.
		+	98 ± 12
(200)		0	N.A.
		+	107 ± 13
RLPA (500)	4	0	96 ± 10 } n.s.
		+	103 ± 8
OLPA (500)		0	83 ± 9 } n.s.
		+	76 ± 13

Legend to table 4.9

* in SC MEM

[1] RLPA = reduced lipoic acid

(a) $P < 0.005$, n.s. $P > 0.05$

[2] compare to treatment with H_2O_2 alone

[3] OLPA = oxidised lipoic acid

[4] temperature of pre-incubation

N.A. = not available

in scheme I.

Pre-exposure to DFO for 24 hours protected cells from the effect observed with ascorbate (table 4.10). This protection increased in a dose-dependent manner and complete protection was evident at a dose of 200 μM (fig 4.22).

4.3.2.10 Effect of zinc salts

To test whether iron could possibly be substituted by other transition metals the potential protective effect of zinc was investigated.

Details of preparation of zinc salt solutions are given in section 4.2. In these experiments only zinc histidine (ZnHis) and zinc aspartate (ZnAsp) were used. Cells were pre-incubated with ZnHis or ZnAsp at concentrations between 100 μM and 1 mM for 2 hours in PBSi at 37°C. This was followed by washing and reincubating in PBSi at 37°C containing 50 μM H_2O_2 for 60 minutes. In each case appropriate controls were prepared. Survival was estimated by the delayed ^3H-TdR incorporation assay as described previously.

Zinc salts at high concentrations exert a cytotoxic action independent of H_2O_2. However, low concentrations show some protection against H_2O_2-induced cytotoxicity. Table 4.11 shows the S.I. of cells in the presence and absence of H_2O_2 following pre-incubation with zinc-histidine. The S.I. in the presence of 100μM H_2O_2 and 50 μM zinc-histidine is significantly different from H_2O_2

SCHEME I

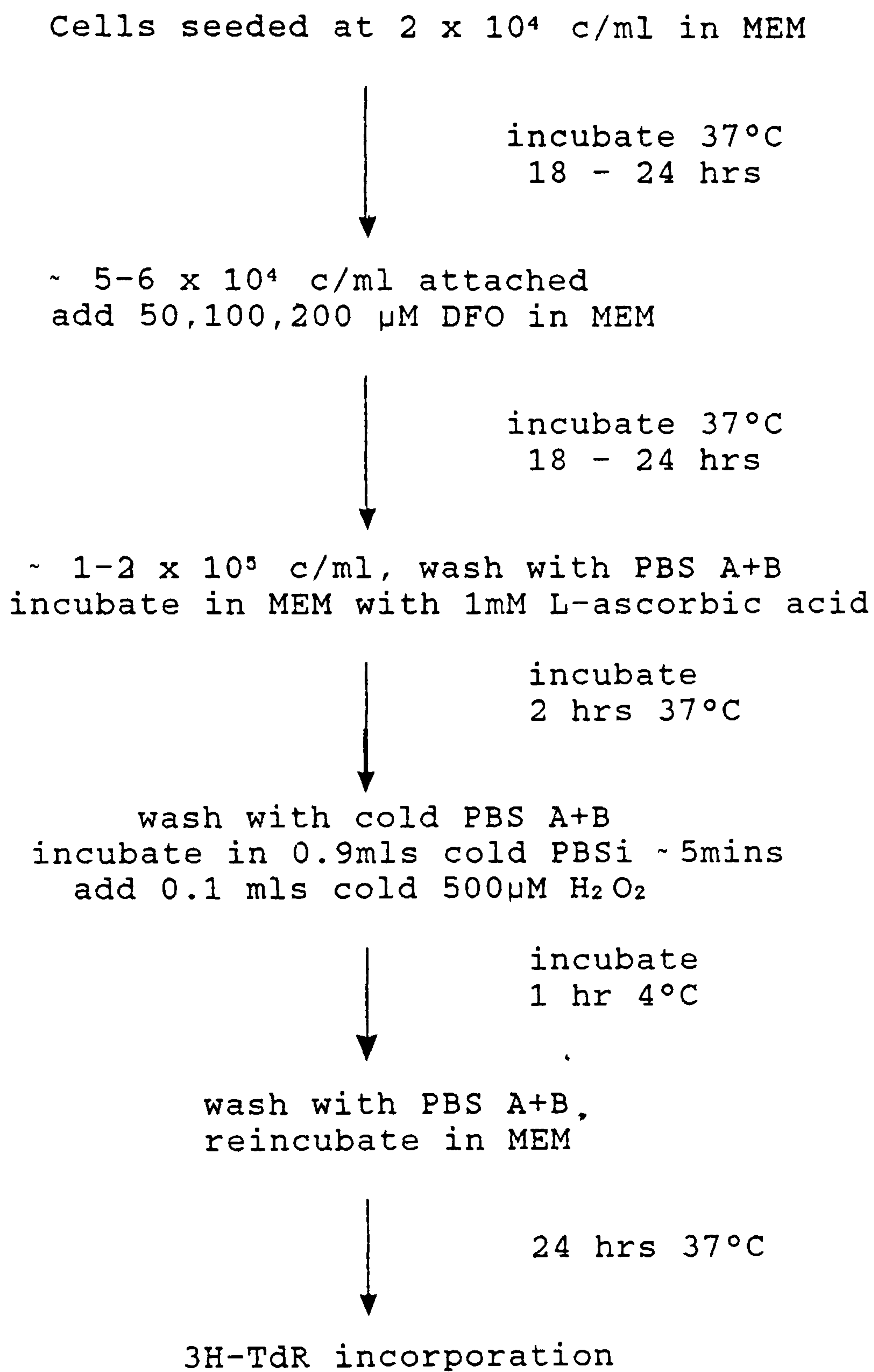


TABLE 4.10

EFFECT OF DESFERRIOXAMINE (DFO) ON THE RESTORATION OF H_2O_2-INDUCED CYTOTOXICITY AT 4°C BY ASCORBATE

Pre-exposure to DFO 24 hrs 37°C (μM)	Pre-incubation with ascorbate 120' 37°C (1mM)	Exposure to H_2O_2 60' 4°C (50μM)	% S.I. ± S.D.	
0	0	0	100 ± 11	n.s.
0	0	+	116 ± 16	
0	+	0	109 ± 20	(a)
0	+	+	46 ± 8	
200	0	0	100 ± 15	n.s.
200	0	+	80 ± 14	
200	+	0	83 ± 18	n.s.
200	+	+	112 ± 22	

(a) P< 0.005

Cells seeded at a density of 2 x 10^4 c/ml were exposed to DFO in SC MEM subsequent exposures were preceded by washing twice with PBS A+B. Ascorbate in SC MEM, H_2O_2 in PBSi.

Legend to fig 4.22:

% S.I. is expressed as a difference between cells incubated with H_2O_2 and cells incubated with H_2O_2 which were previously incubated with DFO.
$5x10^4$ c/ml treated with DFO for 18-24 hrs in SC MEM at 37°C, followed by 50μM H_2O_2 in PBSi pH 7.40 at a cell density of $1.5x10^5$ c/ml.

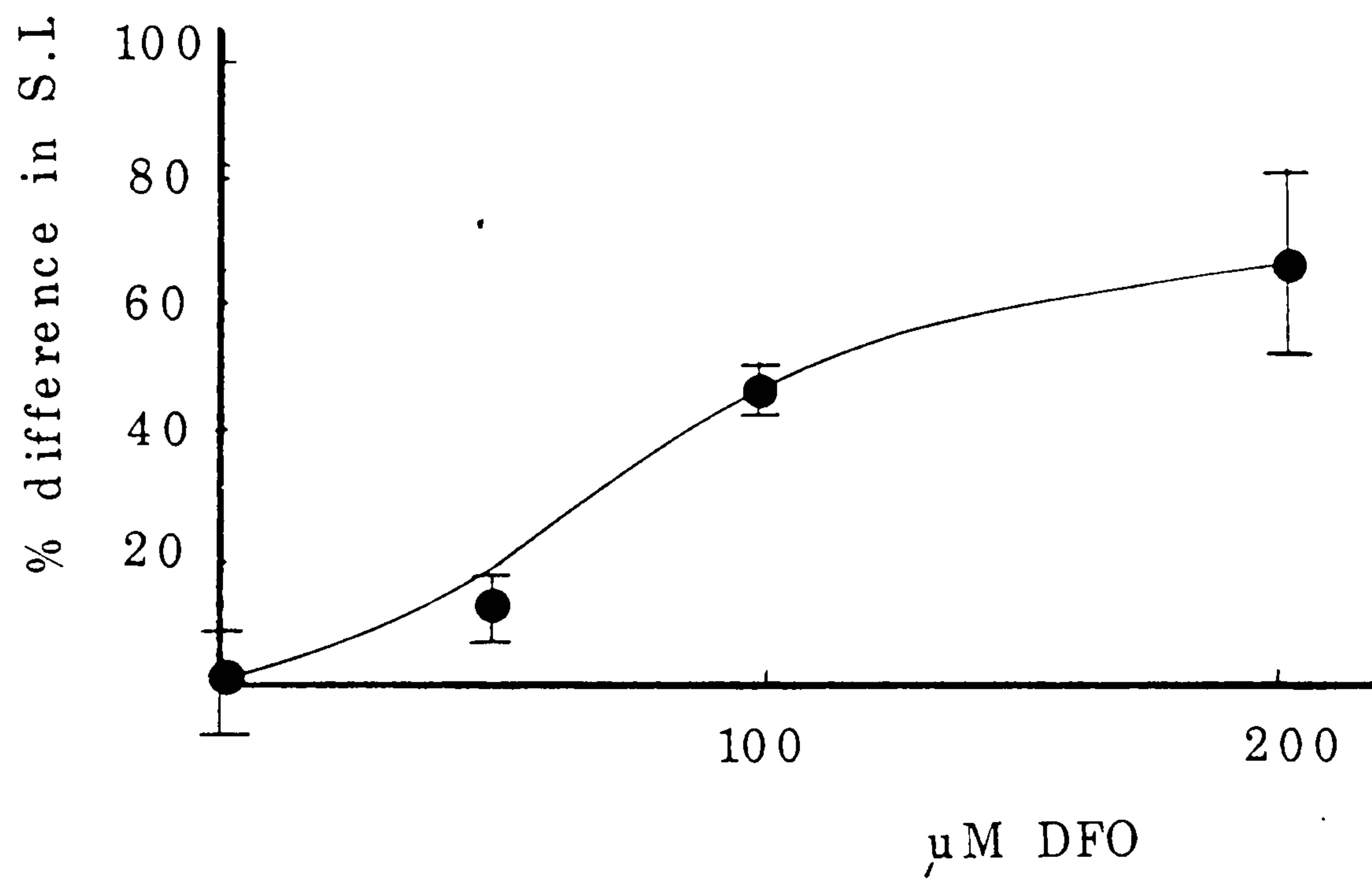


fig 4.22

TABLE 4.11

EFFECT OF ZINC HISTIDINE ON THE %S.I. IN THE PRESENCE OF H_2O_2

ZnHis (μM)*	H_2O_2 (100 μM)	%S.I.
0	-	100 ± 10
0	+	26 ± 5
100	-	77 ± 9
100	+	67 ± 7
500	-	65 ± 9
500	+	15 ± 7
1000	-	10 ± 4
1000	+	4 ± 2

Statistical comparisons (brackets in the %S.I. column): 100 ± 10 vs 26 ± 5 (a); 26 ± 5 vs 67 ± 7 (a); 77 ± 9 vs 67 ± 7 n.s.; 65 ± 9 vs 15 ± 7 (a); 10 ± 4 vs 4 ± 2 (b)

(a) $P<0.01$ (b) $P<0.05$

* cells were pre-incubated with ZnHis in PBSi for 2hrs

treatment alone. This is illustrated in fig 4.23. Zinc aspartate at low concentrationsis even more effective in protecting cells against the effect of H_2O_2 than zinc histidine (table 4.12 and fig. 4.24). Protection by either zinc salt is not complete as the toxicity of higher concentrations of zinc obscures any protective effect.

4.3.3 Modification of hydrogen peroxide-induced cytotoxicity by iron complexes

To investigate the direct involvement of iron several experiments were carried in which cells were exposed to additional iron prior to or during treatment with H_2O_2 using both P.E. and ^3H-TdR incorporation assays as measures of survival.

4.3.3.1 Effect of Fe/EDTA on H_2O_2 cytotoxicity estimated by plating efficiency

Fe(II)/EDTA was made up in a ratio of 1:1 as a stock of 10 mM in distilled water by diluting 1 in 2 of 20mM $FeSO_4$ with 1 in 2 of 20mM EDTA. The complex was prepared immediately before addition to cells. A cell suspension of 2 x 10^6 cells/ml was divided and the aliquots centrifuged at low rpm (800) and the medium carefully removed. The cells were resuspended in 1 ml PBSi containing 100 μM of the Fe/EDTA complex.
Controls were exposed to PBSi alone. The cells were carefully resuspended a few times before removing 0.1 ml into small polypropylene tubes with 1.9 mls of PBSi

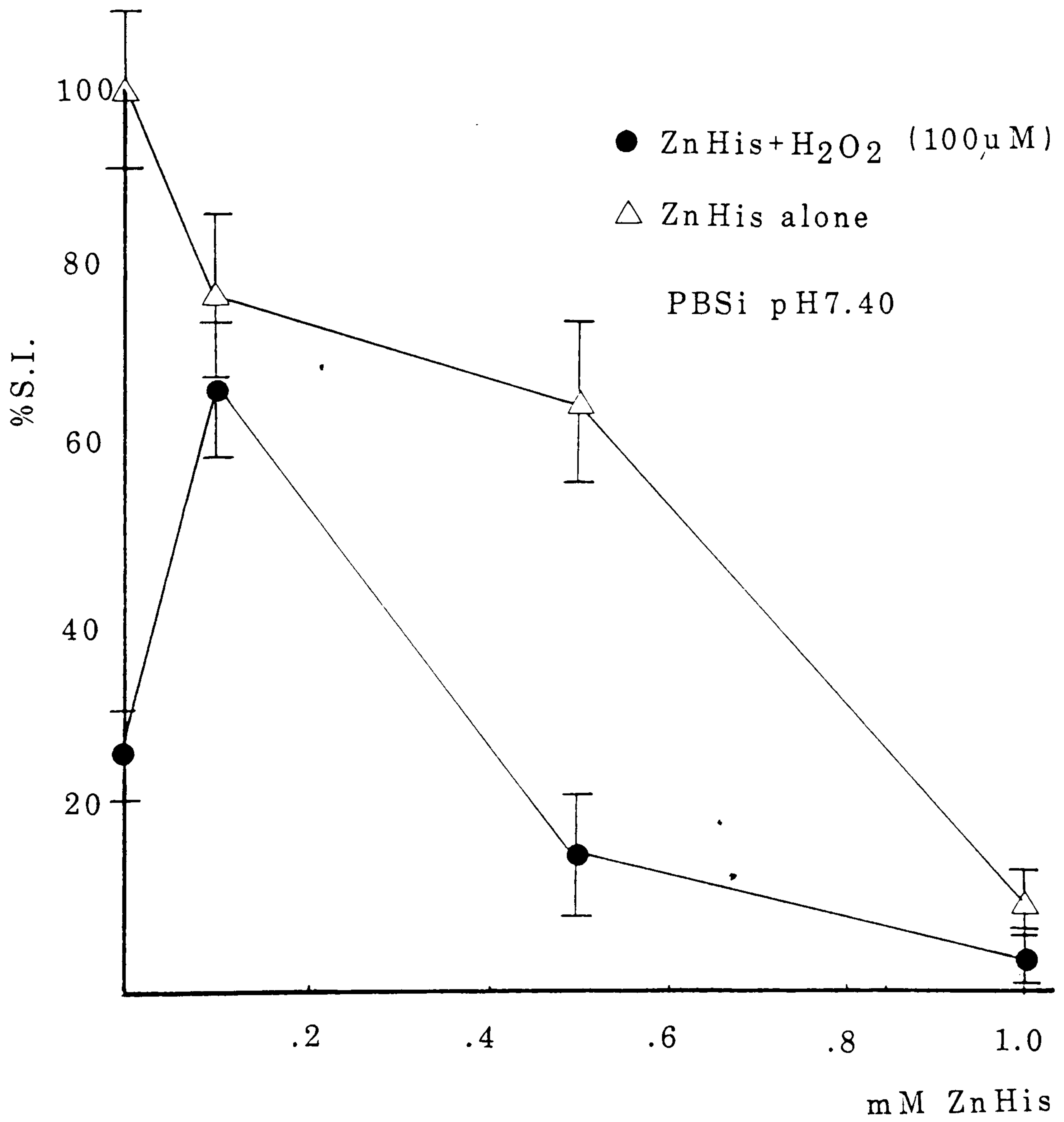


fig 4.23

TABLE 4.12

EFFECT OF ZINC ASPARTATE ON THE %S.I. IN THE PRESENCE OF H_2O_2

ZnAsp (μM)*	H_2O_2 (100 μM)	%S.I. ± S.D.
0	-	100 ± 10
0	+	26 ± 5
50	-	92 ± 16
50	+	69 ± 14
100	-	109 ± 11
100	+	60 ± 6
500	-	70 ± 11
500	+	50 ± 5
1000	-	58 ± 9
1000	+	45 ± 2

Significance brackets: 0− vs 0+ (a); 50− vs 50+ n.s.; 100− vs 100+ (a); 500− vs 500+ (b); 1000− vs 1000+ (b); 0+ vs 50+ (a); 0+ vs 100+ (a); 0+ vs 500+ (a); 0+ vs 1000+ (a)

(a) $P<0.01$ (b) $P<0.05$

* cells were pre-incubated with ZnAsp in PBSi for 2hrs

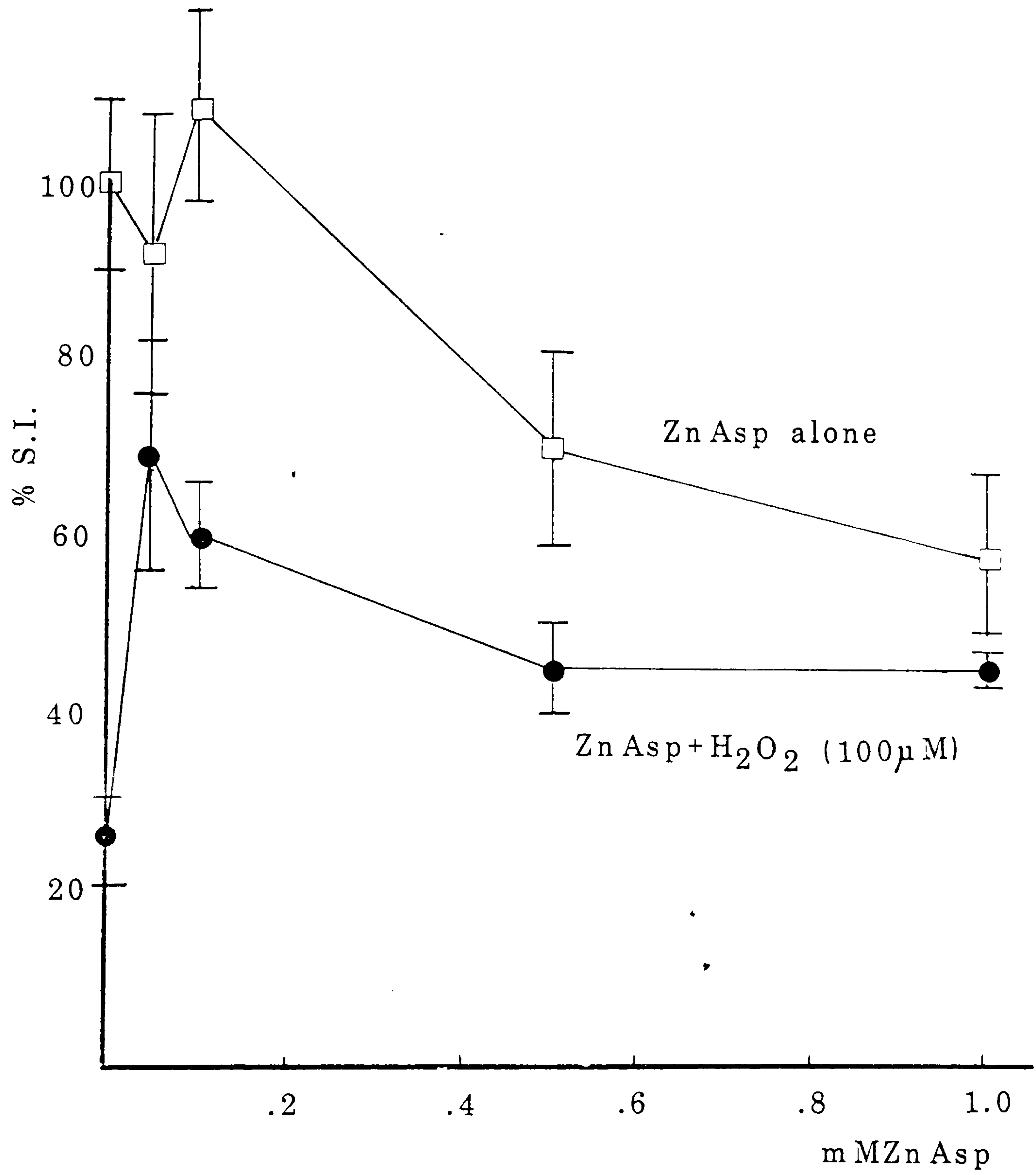


fig 4.24

containing different concentrations of H_2O_2. This suspension was left on a rolling machine (Denley Spiramix) for one hour. The plating efficiency was carried out as described in section 3.2.11. The final concentration of the Fe/EDTA complex was 5μM.

Cells which were exposed simultaneously to 5μM Fe(II)/EDTA (1:1, prepared from $FeSO_4$) and H_2O_2 are protected from damage by H_2O_2 at low concentrations (fig.4.25). In the absence of H_2O_2, Fe(II)/EDTA results in a 35% reduction in survival. Upon addition of low concentrations of H_2O_2 (<10μM) the cells were completely protected by 5 μM Fe(II)/EDTA. Above 10 μM H_2O_2 a reduced toxicity in the presence of Fe(II)/EDTA was found. If only a maximium of 5μM Fe(II) was initially present, it seems surprising that this can protect against 10μM H_2O_2 even if no autoxidation would have taken place. It would be expected that a 1:1 stoichiometry would result in 100% protection against 5μM Fe(II)/EDTA and 5μM H_2O_2. This may be due to the fact that under these conditions very low concentrations of H_2O_2 were rapidly degraded before addition to cells and hence this may have resulted in an overestimation of the concentration of H_2O_2 at the time of the experiment.

4.3.3.2 Effect of pre-incubation with iron dextran (Fe/dex) on plating efficiency

Cells were seeded in small flasks at 5 x 10^4 cells/ml and incubated for 24 hours. The medium was

Legend to fig 4.25:

Survival of CNCM-221 cells estimated by P.E.
$5x10^4$ c/ml
PBSi pH 7.40
incubation time 1hr in suspension.

EFFECT OF Fe(II)/EDTA AND H_2O_2 ON CELL SURVIVAL

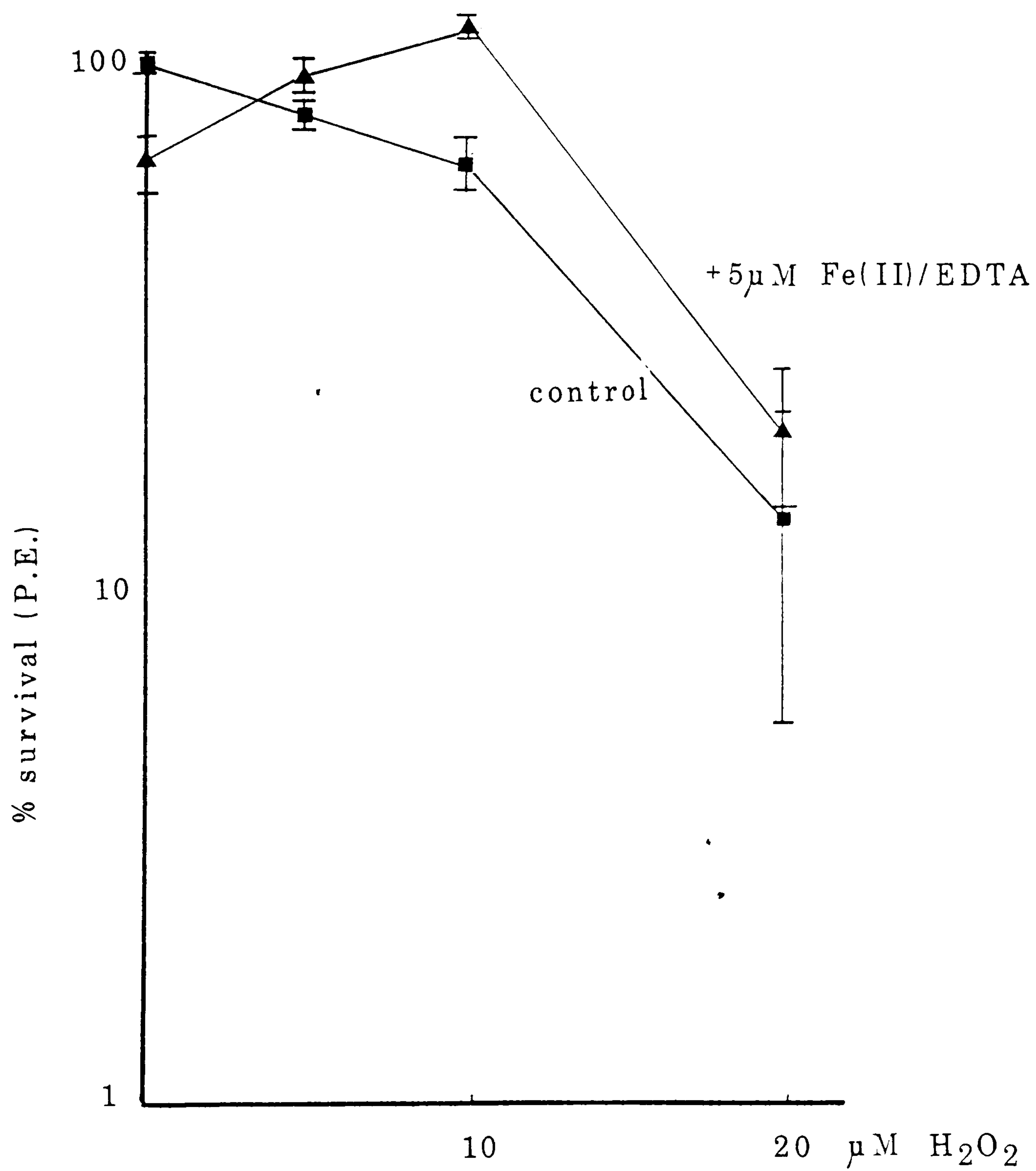


fig 4.25

replaced by PBSi (controls) or PBSi containing 100 μM (equivalent Fe) Fe/dex (batch DA 300P) and the cells incubated for 2 hours at 37°C. After incubation cells were removed by 2% trypsin, resuspended in 2 mls of medium and left for a few minutes. Resuspension was repeated in 1 ml of PBSi and the cells counted in a Coulter counter. Sufficient PBSi was added to give a dilution of 10^6 cells/ml. 0.1 ml was removed and transferred to 2 ml polypropylene tubes containing 0, 30 or 50 μM H_2O_2 in a total volume of 2 mls. After 1 hour incubation at 37°C the cells were processed for the P.E. assay as described in section 4.3.3.1.

Pre-incubation of cells for 2 hours with Fe/dex (batch DA 300P, 100μM Fe) enhanced the cytotoxicity of subsequent exposure to H_2O_2 (table 4.13). The effect is most marked in cells exposed to 30 μM H_2O_2.

4.3.3.3 Effect of iron complexes on H_2O_2 cytotoxicity (^{3}H-TdR incorporation)

The S.I. of cells incubated either in the presence of both iron complexes and H_2O_2 or pre-incubated with iron complexes prior to exposure to H_2O_2 was investigated.

Fe/complexes were added to cells in multiwell dishes either in the presence of H_2O_2 or the cells were pre-incubated with Fe/complexes, washed and the medium replaced by PBSi containing H_2O_2. Incubation in H_2O_2 was carried out for 1 hour at 37°C prior to washing,

TABLE 4.13

INCREASED CYTOTOXICITY OF H_2O_2 IN THE PRESENCE OF Fe/DEX

Pre-incubation Fe/dex (100μM)	H_2O_2 (μM)	% survival (P.E.)	
-	0	100 ± 5	} n.s.
+	0	121 ± 9	
-	30	59 ± 3	} (a)
+	30	33 ± 1	
-	50	9.8 ± 4	} n.s.
+	50	4.4 ± 1	

(a) p = < 0.00025

incubation in growth medium and processing for the ^{3}H-TdR incorporation assay.

4.3.3.4 Simultaneous exposure to iron complexes and H_2O_2

The experimental protocol is outlined in scheme II. The addition of certain iron complexes resulted in a slightly reduced survival in the absence of H_2O_2. The S.I. of cells exposed to 50 μM H_2O_2 alone was 38%. Table 4.14 summarizes the effects.

A positive value for the enhancement factor (E.F.) indicates an enhancement of the effect of H_2O_2, whereas a negative value represents protection. The calculation of E.F. takes into account any effect due to the agent alone. From these data it is clear that both Fe/dex and Fe(II)/8-HQ potentiate the toxicity of H_2O_2, whereas Fe(II)/ATP, Fe(II)/ADP and Fe(II)/EDTA are protective. For comparable iron concentrations the potentiation by Fe(II)/8-HQ is about twofold greater than for Fe/dex. The result with Fe(II)/EDTA is consistent with the data in fig 4.25.

4.3.3.5 Pre-incubation of iron complexes

The protocol is described by scheme III. Incubations with Fe/complexes and H_2O_2 were carried out separately and at either 37°C or 4°C. Appropriate controls were included.

4.3.3.5.1 Effects of iron dextran

The batches used were principally of the Imferon type (those routinely used for intraveneous infusion)

SCHEME II

Cells attached in multiwells ~ 1.5×10^5 c/ml in 1 ml MEM

↓

wash 2x in PBS A+B

↓

1ml PBSi pH 7.40
containing Fe/complex (100μM:500μM)
add 50μM H_2O_2
incubate for 1 hour at 37°C

↓

wash 2x in PBS A+B

↓

1 ml MEM 24 hours 37°C

↓

add 1 μCi ^{3}H-TdR 30 minutes

↓

wash; fix; digest

↓

scintillation counter

TABLE 4.14

EFFECTS OF IRON COMPLEXES ON THE CYTOTOXICITY OF H_2O_2

(100μM Fe, 500μM LIGAND)

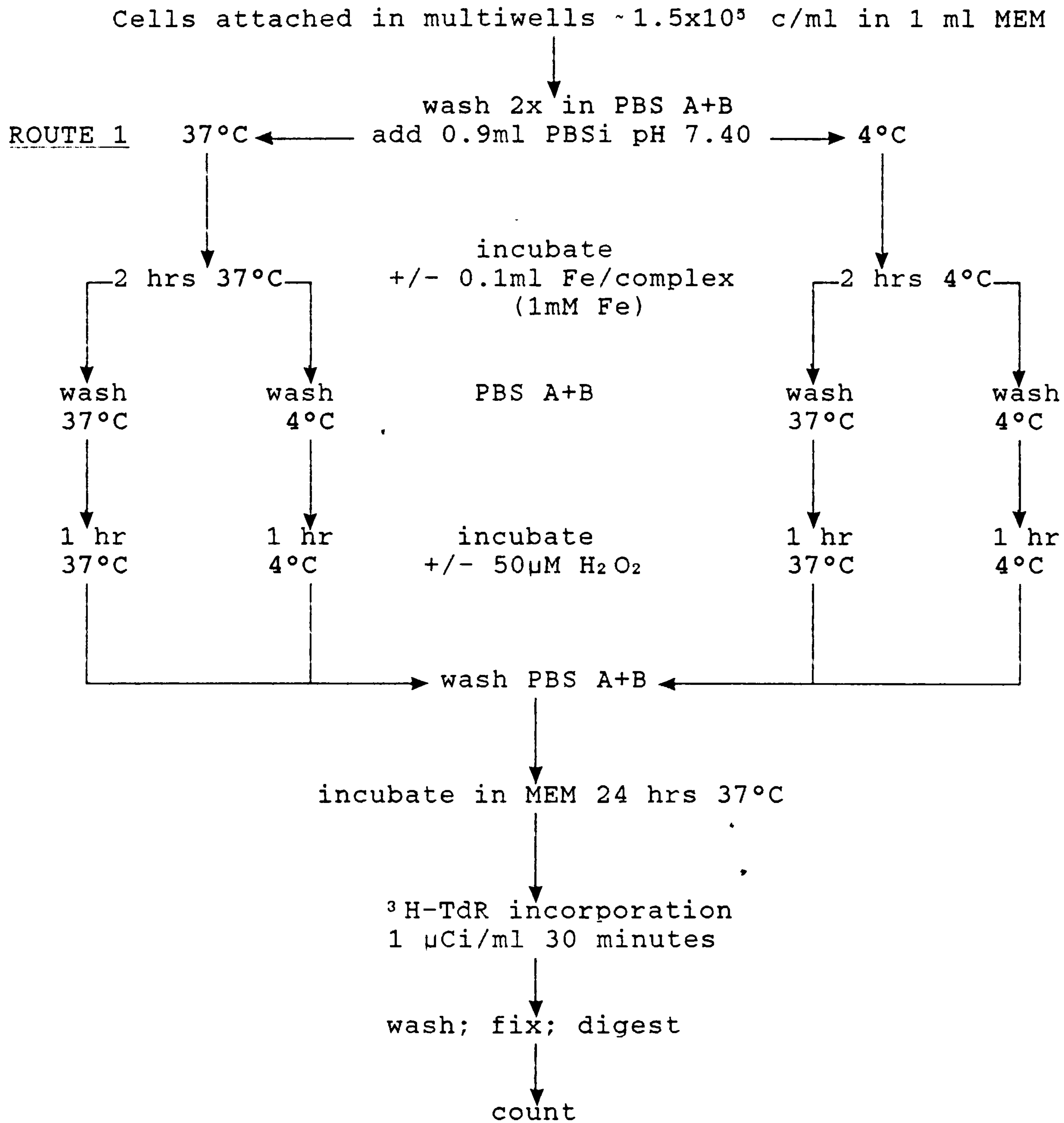
Agent 1Fe:5Lig	H_2O_2 (50μM)	%S.I. ± S.D.		Enhancement Factor % (E.F.)*
-	0	100 ± 8	} (a)	-
-	+	38 ± 9		
8-HQ/Fe(II)	0	18 ± 1	} (a)	+ 35
"	+	0.5 ± 0	(a)[2]	
Fe/dex[1]	0	79 ± 11	} (a)	+ 19
"	+	15 ± 10	(a)[2]	
EDTA/Fe(II)	0	81 ± 10	} (b)	- 26
"	+	52 ± 10	n.s.[2]	
ADP/Fe(II)	0	72 ± 25	} n.s.	- 87
"	+	90 ± 19	(a)[2]	
ATP/Fe(II)	0	74 ± 23	} n.s.	- 97
"	+	100 ± 14	(a)[2]	

* E.F. = (S.I. H_2O_2/S.I. control) - (S.I. agent + H_2O_2/S.I. agent)

(a) $P<0.01$ (b) $P<0.05$

[1] Fe/dex = iron dextran (not 1:5 ratio)
[2] compared to treatment with H_2O_2 alone

SCHEME III



with a standard iron content. Cells were tested at a density of 1.5×10^5 cells/ml. Pre-incubation of cells with Fe/dex (batch BM 324s) and exposure to 50 µM H_2O_2 were carried out under different conditions of temperature (table 4.15).

When both incubations were carried out at 37°C a significant increase in toxicity following pre-incubation with Fe/dex was observed. When the pre-incubation was performed at 4°C increased H_2O_2-induced toxicity was prevented and the reduction in survival is analogous to that of cells treated with H_2O_2 alone at 37°C. Exposure to Fe/dex and H_2O_2 at 4°C results in no significant difference from the untreated controls. A small increase in toxicity was observed when cells were pre-incubated with Fe/dex at 37°C, followed by H_2O_2 at 4°C but this appears to be due to the effect of the Fe/dex alone. Provided there is no diffusion barrier to H_2O_2 at 4°C a reaction can still occur intracellularly provided pre-incubation with Fe/dex occurred at 37°C. However, the toxicity at 4°C was increased only by 28% compared to the untreated control in contrast to 82% for treatment at 37°C when compared to the untreated control.

A number of different batches of Fe/dex were tested for their ability to potentiate the toxicity of H_2O_2. The results are shown in table 4.16. The greatest effect was produced by batch DA 300P which caused a 56% decrease in survival compared to H_2O_2 alone (30%). Following pre-

TABLE 4.15

EFFECT OF PRE-INCUBATION WITH Fe/DEX ON H_2O_2-INDUCED CYTOTOXICITY

Agent (100μM Fe)	T°C of pre-incubation	Addition of 50μM H_2O_2	T°C of incubation	% S.I. ± S.D.
–	37	–	37	100 ± 17
				} (a)
–	"	+	"	46 ± 14
BM 324s	"	–	"	72 ± 24 (b)
				} (b)
"	"	+	"	18 ± 10
–	4	–	"	100 ± 20
				} (a)
–	"	+	"	42 ± 7
BM 324s	"	–	"	100 ± 9 n.s.
				} (a)
"	"	+	"	44 ± 9
–	37	–	4	100 ± 5
				} n.s.
–	"	+	"	104 ± 18
BM 324s	"	–	"	84 ± 10 (b)
				} n.s.
"	"	+	"	74 ± 6
–	4	–	4	100 ± 18
				} n.s.
–	"	+	"	90 ± 15
BM 324s	"	–	"	102 ± 18 n.s.
				} n.s.
"	"	+	"	108 ± 17

(a) $P < 0.01$ (b) $P < 0.05$

TABLE 4.16

EFFECT OF BATCHES OF IRON DEXTRAN ON THE CYTOTOXICITY OF H_2O_2

Fe/dex batch (100 μM Fe)	H_2O_2 (30μM)	% S.I. ± S.D.	
-	-	100 ± 19	(b)[1]
-	+	67 ± 5	
DA 1M	+	51 ± 9	(b)[2]
DA 16H	+	49 ± 8	(b)
DA 3H	+	50 ± 8	(b)
CM 2K2	+	46 ± 14	(b)
BM 324s	+	54 ± 8	n.s.
DA 300P	+	39 ± 6	(a)
DA 301s	+	53 ± 13	n.s.
Imposil	+	52 ± 10	n.s.
Gleptosil	+	53 ± 4	(b)
DA 4K1	+	58 ± 10	n.s.

(a) $P<0.01$ (b) $P<0.05$

[1] compared to control
[2] compared to treatment with H_2O_2 alone

incubation with batch DA 300P for 4 hours in serum-containing medium cells were exposed to increasing concentrations of H_2O_2 (fig 4.26). The data show a toxic effect of the Fe/dex alone (25% reduction in S.I.) and substantial enhancement of H_2O_2 cytotoxicity.

4.3.3.5.2 Effect of Fe/ATP

Pre-exposure of cells to Fe(III)/ATP for 2 hours at either 4°C or 37°C did not modify the toxicity of H_2O_2 (table 4.17).

4.3.3.5.3 Effect of Fe/8-HQ

Fe/8-HQ exerts a toxicity by itself which increases with increasing concentration of the complexing agent. This was shown in section (3.3.4.1, Chapter III). However, the concentrations used in these experiments were 50 µM $FeCl_3$ complexed to 100 µM 8-HQ which shows relatively low toxicity in the absence of H_2O_2. Cells pre-exposed to Fe/8-HQ were markedly sensitized to H_2O_2 (fig 4.27). This enhancement of H_2O_2-induced cytotoxicity is much greater than that produced by pre-exposure to equivalent amounts of iron as Fe/dex.

4.3.3.6 Effect of pre-incubation with zinc aspartate

In section 4.3.2.10 it was shown that zinc salts partially protected cells from H_2O_2. Analogous experiments were performed to test whether zinc can protect cells from the Fe/dex potentiation of H_2O_2-induced toxicity.

The procedure used was analogous to that outlined in

Legend to fig 4.26:

$1.5x10^5$ c/ml of CNCM-221 cells pre-incubated with 100μM Fe/dex (batch DA 300P) for 4 hrs in SC MEM, followed by H_2O_2 for 1 hr in PBSi pH 7.40.

$\underline{\text{EFFECT OF Fe/DEX AND } H_2O_2 \text{ ON THE S.I. OF CELLS}}$

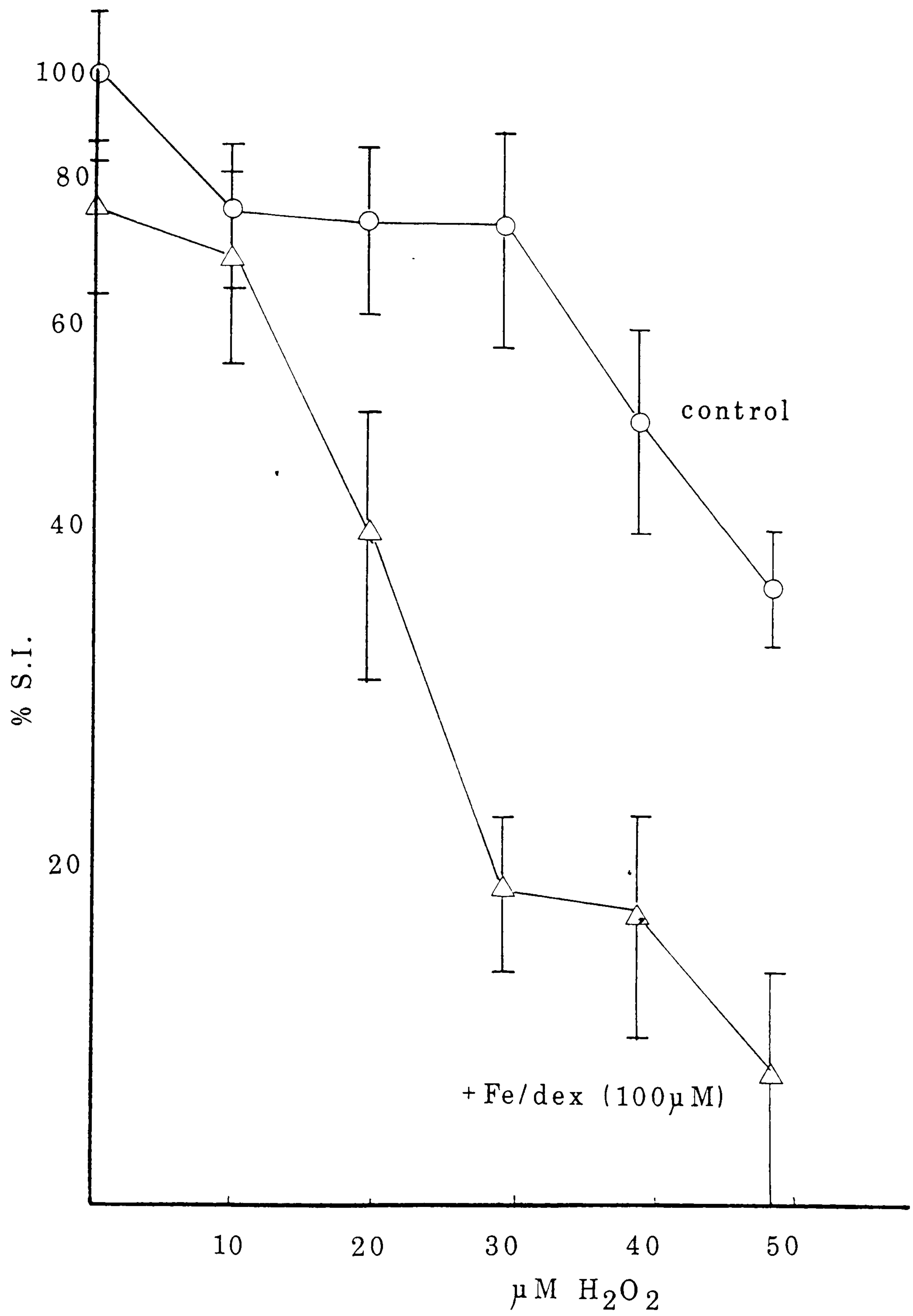


fig 4.26

TABLE 4.17

EFFECT OF PRE-INCUBATION WITH Fe/ATP ON THE H_2O_2-INDUCED CYTOTOXICITY

Agent*	Preincubation T°C	50µM H_2O_2	Incubation T°C	%S.I. ± S.D
-	37	-	37	100 ± 20
				} (a)
-	"	+	"	47 ± 16
Fe/ATP	"	-	"	100 ± 14 n.s.
				} (a)
"	"	+	"	38 ± 5
-	4	-	"	100 ± 18
				} (a)
-	"	+	"	38 ± 7
Fe/ATP	"	-	"	78 ± 11 n.s.
				} (a)
"	"	+	"	41 ± 3
-	37	-	4	100 ± 9
				} n.s.
-	"	+	"	103 ± 11
Fe/ATP	"	-	"	124 ± 14 n.s.
				} n.s.
"	"	+	"	116 ± 27

* 100 µM $FeCl_3$: 500µM ATP

(a) $P < 0.01$

Legend to fig 4.27:

Pre-incubation of $1.5x10^5$ c/ml (CNCM-221) with Fe(III)/8-HQ (50μM:100μM) for 2 hrs in PBSi pH 7.40 followed by H_2O_2 for 1 hr in PBSi pH 7.40.

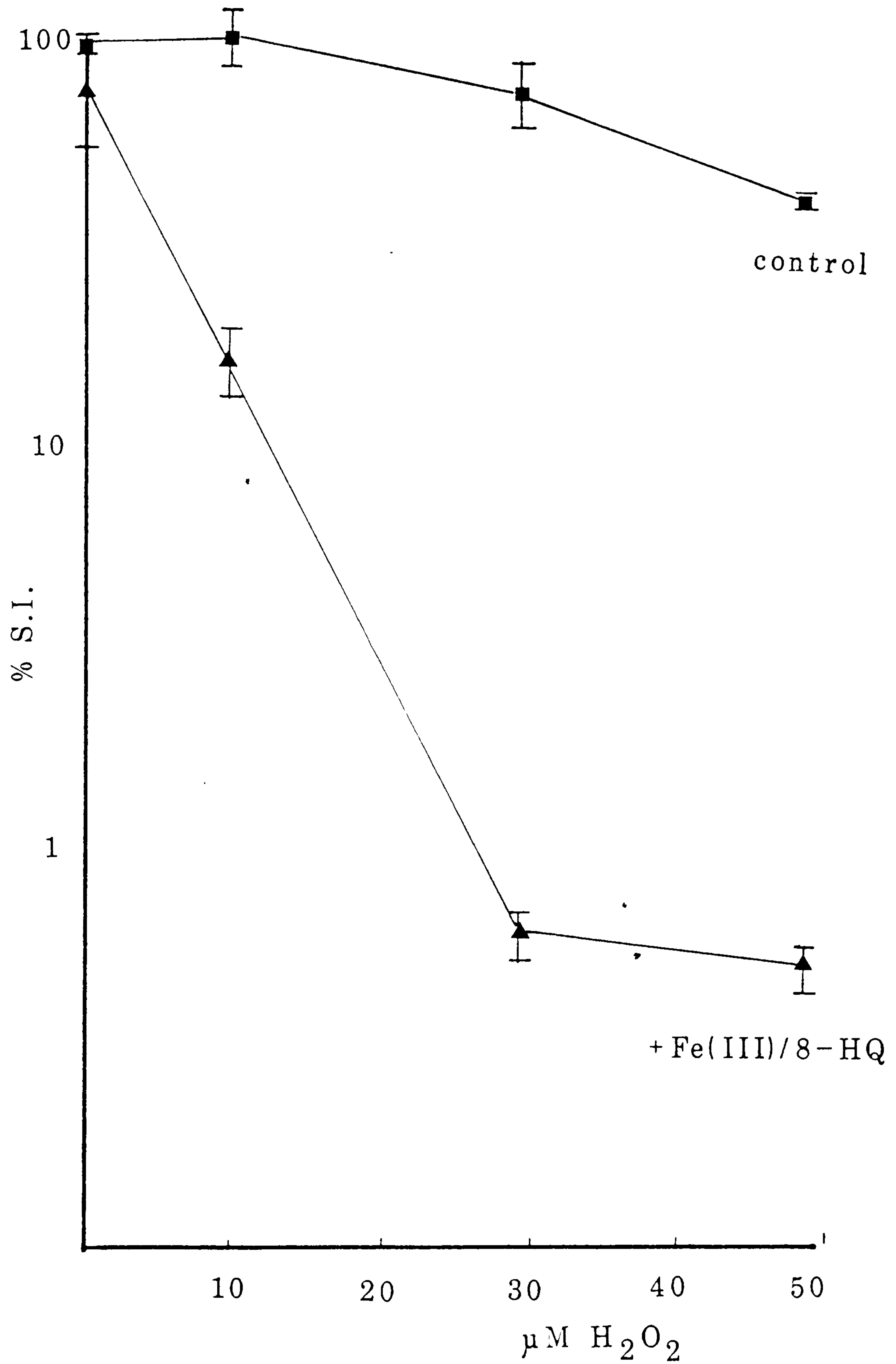


fig 4.27

scheme III (route 1). The additional step to this was that prior to incubation with Fe/dex the cells were pre-incubated with zinc aspartate at different concentrations in medium for 2 hours at 37°C, washed and re-incubated for 2 hours with Fe/dex. Incubation with H_2O_2 was carried out at 37°C. Cytotoxicity was measured by the delayed ^3H-TdR incorporation assay.

The results are shown in table 4.18. The survival with H_2O_2 alone was 58%. Fe/dex enhanced this toxicity by 10%. This enhanced effect was diminished by 22% when cells were pre-incubated with zinc aspartate.

4.3.3.7 Pre-incubation with Fe/dex treated with ascorbate at 60°C

In chapter II, section 2.3.2.6 it was demonstrated that heating Fe/dex with ascorbate leads to almost complete reduction of all the iron present. It was therefore employed to test the effect of using the reduced form of Fe/dex on H_2O_2-induced cytotoxicity.

Fe/dex was heated (60°C) with ascorbate in excess (5 mM ascorbate to 1 mM Fe/dex) for 30 minutes to reduce the iron in the dextran. The material was allowed to cool and then filter-sterilised (the filters used have a pore diameter of 0.2 μm; iron dextrans are 13 nm in diameter) and pre-incubated with cells at 37°C for 1 hour in PBSi. The cells were washed with cold PBS A+B and exposed to 50 μM H_2O_2 for 1 hour at 4°C in cold PBSi. Appropriate controls were included. Cytotoxicity was estimated by the

TABLE 4.18

EFFECT OF ZINC ASPARTATE ON THE IRON DEXTRAN ENHANCED H_2O_2 CYTOTOXICITY

PRE-INCUBATIONS			
1 ZnAsp (200μM) 2 hrs 37°C	2 Fe/dex (100μMFe) 1.5 hrs 37°C	2 H_2O_2 (50μM) 1 hr 37°C	%S.I. ± S.D.
-	-	-	100 ± 17 } (a)
-	-	+	58 ± 9
-	+	-	109 ± 14 } (a)
-	+	+	48 ± 9 ⎤ (b)
+	-	-	105 ± 16 } n.s.
+	+	-	115 ± 17
+	+	+	70 ± 12 ⎦ (b)

(a) P<0.01 (b) P<0.05

[1] Cells (1.5×10^5 c/ml) were pre-incubated with zinc aspartate in serum-containing medium.

[2] Incubation in PBSi

delayed ^{3}H-TdR incorporation assay.

Cells exposed to this reduced form of Fe/dex followed by incubation with H_2O_2 at 4°C exhibited the cytotoxicity to a significantly higher degree than pre-incubation with the appropriate pre-heated ascorbate or pre-heated Fe/dex controls, as shown in table 4.19.

TABLE 4.19

RESTORATION OF THE TOXICITY OF H_2O_2 AT 4°C BY PRE-INCUBATION WITH PRE-HEATED ASCORBATE AND Fe/DEXTRAN

Agent* pre-incubation 37°C	H_2O_2 (50μM) 4°C	% S.I. ± S.D.
-	-	100 ± 8
-	-	93 ± 7
Fe/dex(100μM)	-	90 ± 9
"	+	74 ± 9
Asc. (500μM)	-	95 ± 14
"	+	42 ± 10
Fe/dex + Asc	-	105 ± 4
"	+	19 ± 8

Statistical comparisons (brackets beside the % S.I. column): 100 ± 8 vs 93 ± 7: n.s.; 90 ± 9 vs 74 ± 9: n.s.; 95 ± 14 vs 42 ± 10: (a); 105 ± 4 vs 19 ± 8: (a); 93 ± 7 vs 74 ± 9: (b); 93 ± 7 vs 42 ± 10: (a); 93 ± 7 vs 19 ± 8: (a); 42 ± 10 vs 19 ± 8: (b).

* Agents were heated for 30 mins to 60°C; Fe/dex and ascorbate were heated together. Pre-incubation in PBSi.

(a) $P<0.005$; (b) $P<0.05$

4.4 DISCUSSION

4.4.1. Degradation of H_2O_2 by cells

The dose-response curve for H_2O_2 obtained by the two independent methods used to measure cell viability resulted in a characteristic shoulder effect. This effect has been frequently observed with cells exposed to ionising radiation. Interpretation of these observations has often been associated with delayed repair mechanisms (Alper,1987).

In the case of exposure of H_2O_2 as a source of free radical generation, the shoulder is diminished as the cell density is reduced (see fig. 4.12). This led to the consideration of the capacity of the cell to remove H_2O_2. The rate of degradation of H_2O_2 was found to increase with cell number. The response was biphasic and consisted of an instantaneous removal of 5-10% within the first few minutes. This was followed by a steady rate of degradation during the remaining 60 minutes. Attempts to interfere with the enzymatic systems for removal of H_2O_2 resulted in affecting both phases. Glucose was added as a source of reducing equivalents for GPx. However, despite insignificant effect on the rate of removal of H_2O_2, complete protection of the cells was afforded. This suggests that glucose protects cells by a mechanism which does not involve the enzymatic removal of H_2O_2. One possibility is that it is acting as a scavenger for HO· radicals. HO· radicals reactions with glucose have been

shown to occur at various positions of the carbohydrate (Schuchmann & v.Sonntag,1977,1978b). However, since substantial protection was also afforded when glucose was added 10 minutes after H_2O_2 exposure, a more plausible possibility may be its involvement in a subsequent repair mechanism.

Time-dependent repair of DNA lesions has been previously demonstrated (Olson,1988). It has recently been suggested that one mechanism of cell killing may be due to the time-dependent depletion of cellular energy metabolites, which are rapidly used for the synthesis of poly ADP-ribose for the repair of DNA lesions (Berger & Berger, 1988). Inhibition of the enzyme poly(ADP) ribose transferase has resulted in potentiation of both detectable DNA lesions and cytotoxicity (Cantoni et al.,1987). Glucose may enter the pentose phosphate shunt to generate ribose-5-phosphate for biosynthesis of nucleic acids and thereby participate in the necessary repair mechanism. If this hypothesis is correct, then the predominant damage to the cells must be occurring instantaneously and any subsequent events would be related to time-dependent repair mechanisms. This could be tested by adding an excess of H_2O_2 to cells in PBSi for only 1 minute, followed by replacement of fresh PBSi in the absence of H_2O_2 for the remaining 60 minutes. Only a limited amount of repair could take place in the absence of an energy source and similar damage would be

expected from a 1 minute exposure or a 60 minute exposure.

The proposal that instantaneous removal of H_2O_2 can be attributed to the predominant proportion of the observed damage is consistent with the greater initial rate of removal observed in the presence of PCA which was also reflected an increased cytotoxic effect. This is contrary to the expectation that it may inhibit GPx and thus reduce the rate of H_2O_2 degradation. The sensitivity of PCA for GPx is based on studies carried out with isolated enzymes. It is questionable whether within cells in vitro PCA is specifically targeted towards GPx. As a thiol compound it may provide suitable reducing equivalents for transition metals which would enable instantaneous Fenton reactions to take place. This may account for the observed initial increase in H_2O_2 degradation.

Reducing the rate of degradation of H_2O_2 during the second phase of the incubation appeared to have no effect on the cytotoxicity, as was demonstrated with the addition of 3-ATZ. Thus, although catalase may have been inhibited and it has been demonstrated to be a haem-type enzyme inhibitor (Geffner _et al_.,1987), this effect is nevertheless independent of the instantaneous damage caused by H_2O_2.

It was found that the dependency of survival on cell density following exposure to H_2O_2 was eliminated

when the dose was expressed in terms of f moles/cell. This suggests that the criterion for damage to cells is the number of targets being hit per cell. No correlation between the total dose (expressed as a function of time in µmole mins) and the number of cells was evident, supporting the suggestion that the time of exposure to H_2O_2 is not related to the cytotoxic effect. Yet, the repeated observation that higher cell densities appear to be increasingly resistant to H_2O_2 which is initially in excess is perhaps suprising in the first instance. This anomaly can perhaps be discussed with regard to rates of cell growth. There is well documented evidence that in populations of normal cells a diminuition in the rate of growth is observed as a critical cell density is approached irrespective of the seeding density (Todaro <u>et al</u>.,1965; Castor, 1968; Houck <u>et al</u>., 1972). With the use of ^3H-TdR as a label for growing 3T3 cells at different densities in specially designed slide cases (Riley <u>et al</u>, 1973) it has been shown that an inverse relationship exists between the labelling index and increasing cell density (Canagaratna & Riley, 1975).

To illustrate this pattern of density dependent growth inhibition by CNCM I-221 cells the labelling index has been expressed as a ratio of cpm to the absorbance obtained at 280nm. This optical extinction estimates the total cellular protein and thus reflects the total number of cells. Plotted against the cell density this results

in an inverse relationship (fig 4.28) consistent with previous findings (Canagaratna & Riley, 1975). Thus, the proportion of cycling cells falls with increasing cell density and this phenomenon may be regarded as a sensitivity factor which has a characteristic pattern according to the type of cell. It may be that certain phases of the cell cycle are sensitive to damage by certain agents. It is known, for instance, that many cells in G2 of the cell cycle are particularly sensitive to damage by radiation (Coggle,1983; Trowell,1966). This suggests, that if cells are sensitive to H_2O_2 in any particular stage of the cycle, then a smaller proportion of these cells will be affected in a dense population than in a sparse population. This could be tested by growing synchronous cells and then exposing them to H_2O_2 at different stages of the cycle.

Consistent with the possibility of cycling dependent sensitivity is the observation that the effect of H_2O_2-induced damage on ^3H-TdR incorporation is only expressed 18-24 hours after exposure, suggesting that only cells which were in the cycling phase would be affected.

4.4.2. Mechanism of H_2O_2-induced cytotoxicity

In the context of predominantly instantaneous events occurring the amount of damage could be related to the metabolic state of the cell prior to exposure to H_2O_2. This will determine the amount of reduced metal in

RELATIONSHIP BETWEEN THE RATIO OF CPM TO O.D.$_{280nm}$ AND CELL DENSITY

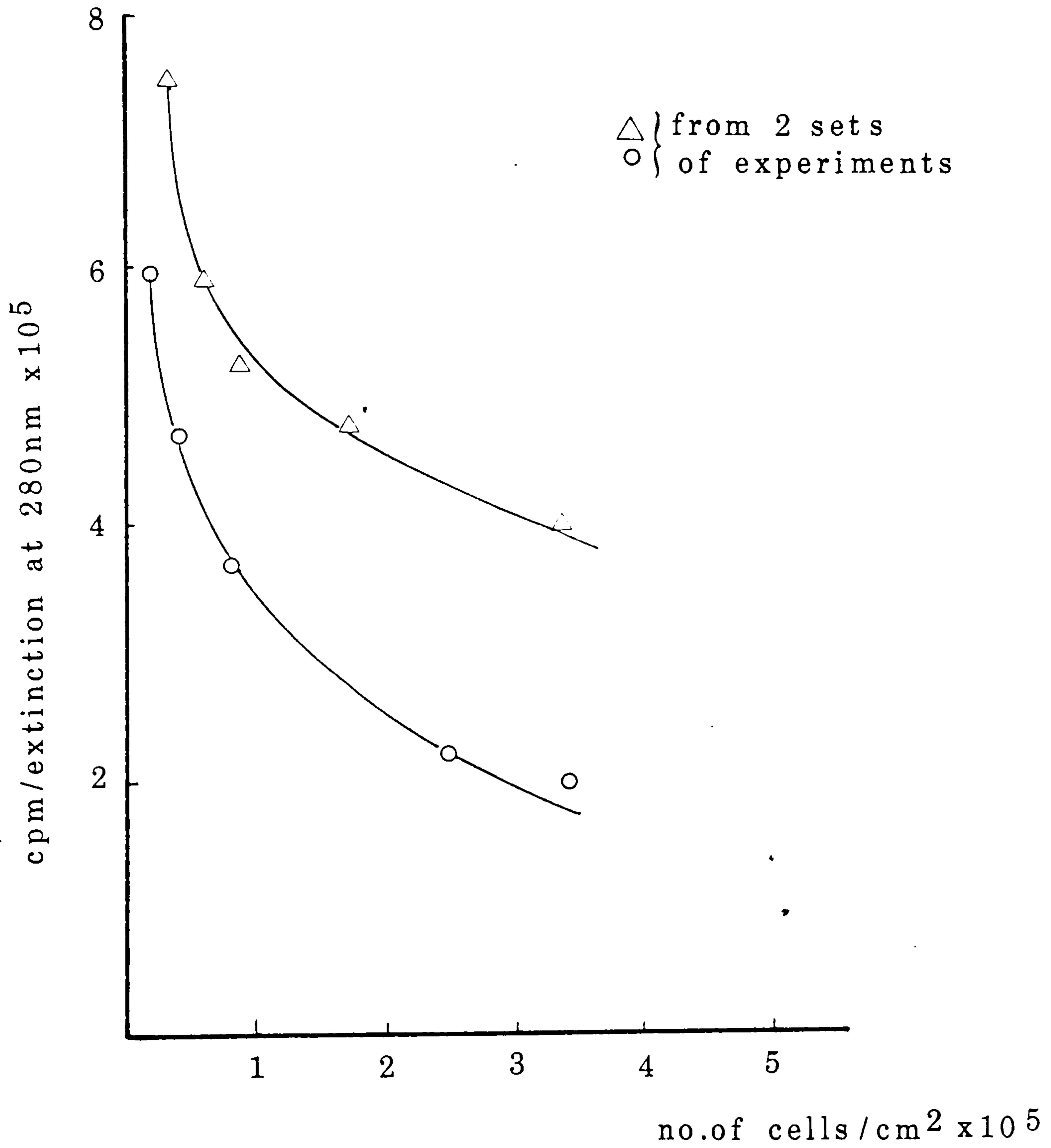


fig 4.28

the cell. At 37°C a significant proportion of reduced metal is available for reaction. If reductive metabolism is temperature dependent then this would be inhibited at 4°C. Inhibition at 4°C is consistent with the observation that no instantaneous removal of H_2O_2 was evident, whereas at 37°C at least 10% of H_2O_2 was removed in less than one minute (see fig 4.10). This initial 10% may reflect the proportion of reduced metal which has reacted with H_2O_2.

The effect of temperature was previously investigated by Ward et al.(1985). In their experiments it was shown that at 0°C both V79 and HeLa cells are not readily killed by concentrations of H_2O_2 upto 20 mM, which at 37°C are lethal. SSB were observed under conditions of low temperature but do not result in cytotoxicity. From fig.4.21 it is evident that for 1.5 x 10^5 cells/ml H_2O_2 concentrations upto of 100μM are not toxic at 4°C. The same concentrations applied at 37°C showed dose-dependent cytotoxicity.

The mechanism of cell killing by H_2O_2 at 37°C is generally attributed to damage to DNA. The types of damage produced can be summarized as outlined in table 4.20. Analogous types of damage have been observed for the HO· radicals produced by ionizing radiation (Ward,1975). In order to generate a sufficiently large radical flux from a relatively small concentration of transition metal ions (eg iron) which will tend to be in

TABLE 4.20

TYPES OF DNA DAMAGE CAUSED BY OXIDATIVE STRESS

Type of damage	treatment conditions	references
base release	0.05M H_2O_2 10μM Fe^{3+} 37°C 5 days	Rhaese 68
depolymerisation	0.04M H_2O_2 100μM Cu^{2+} 37°C 2 days 100μM Fe^{3+}	Schweiz 69
base damage SSB DSB crosslink	0.088M H_2O_2 37°C 20 days	Massie 72
DNA-DNA CL DNA-protein CL (chromatin)		Lesko 82

the oxidised state a reductive process would appear to be necessary. The addition of ascorbate to cells which were subsequently incubated at 4°C with H_2O_2 was shown to restore the cytotoxicity to the same degree as was observed at 37°C. This suggests that ascorbate acts (inter alia) by reducing transition metals within the cell and stimulating the Fenton reaction. At 37°C the source of reducing equivalents is likely to be a metabolic process which is interrupted at 4°C. The requirement for an extended incubation period and the fact that DHA at concentrations of 1mM also restores the cytotoxic effect of H_2O_2 at 4°C if pre-incubation takes place at 37°C suggests that ascorbate autoxidises extracellularly and is taken up by the cells as DHA (fig 4.29). Intracellularly it then becomes metabolically re-reduced to ascorbate. This process has also been suggested by Bridges & Hoffman (1986) who measured the rate of uptake of ascorbate by K562 cells. Cells incubated with [^{14}C]-labelled ascorbate for 2 hours exhibited a linear uptake rate of 0.029 nmol/min/10^7 cells. The half-life of ascorbate oxidation is less than 2 hours (Lewin,1976) and the cells were found to have a high content of ascorbate as measured by HPLC following exposure to DHA. This would explain why DHA was equally effective when applied to cells at 37°C and why it did not restore the cytotoxicity when cells were pre-incubated with DHA at 4°C.

OXIDATION OF ASCORBIC ACID

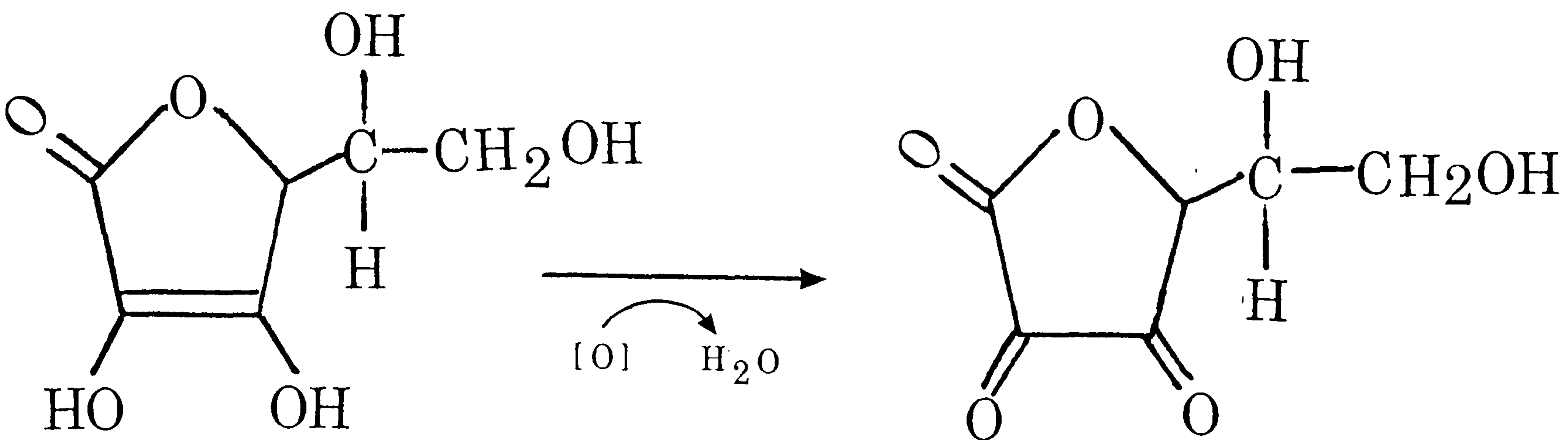


fig 4.29

The restoration of H_2O_2 cytotoxicity at 4°C is abolished by prolonged pre-incubation of cells with DFO. A 24 hour pre-incubation was required as times less than this had no effect, suggesting a slow rate of DFO uptake by cells, or alternatively a slow release of iron from the cell. Low cell uptake of DFO has been reported previously (Halliwell & Gutteridge,1985). DFO specifically binds Fe^{3+} and is known to inhibit superoxide-driven reduction of Fe^{3+} (Halliwell & Gutteridge,1985). It is likely that by chelating Fe^{3+} it will also prevent ascorbate driven reduction. A tentative model is proposed to explain the data (fig. 4.30).

Lipoic acid was less effective in restoring H_2O_2 cytotoxicity at 4°C and at higher concentrations it exerts a toxicity by itself at 37°C. Cells exposed to a concentration of 200μM at 37°C had a survival index of 60%, but at 4°C no damage was evident, even at the higher toxic concentration of 500μM, suggesting that lipoic acid is not taken up by the cells at this temperature (see table 4.9).

The transition temperature of H_2O_2-induced cytotoxicity occurred between 27°C and 22°C, below which the toxic effect was considerably reduced. Transition temperatures for cells may vary considerably according to cell type, eg. studies on the uptake and degradation of substrates by mouse peritoneal macrophages have shown that membrane internalisation was completely inhibited at

SCHEMATIC DIAGRAM OF THE INTRACELLULAR EVENTS LEADING TO THE RESTORATION OF H_2O_2 -INDUCED CYTOTOXICITY AT 4 °C.

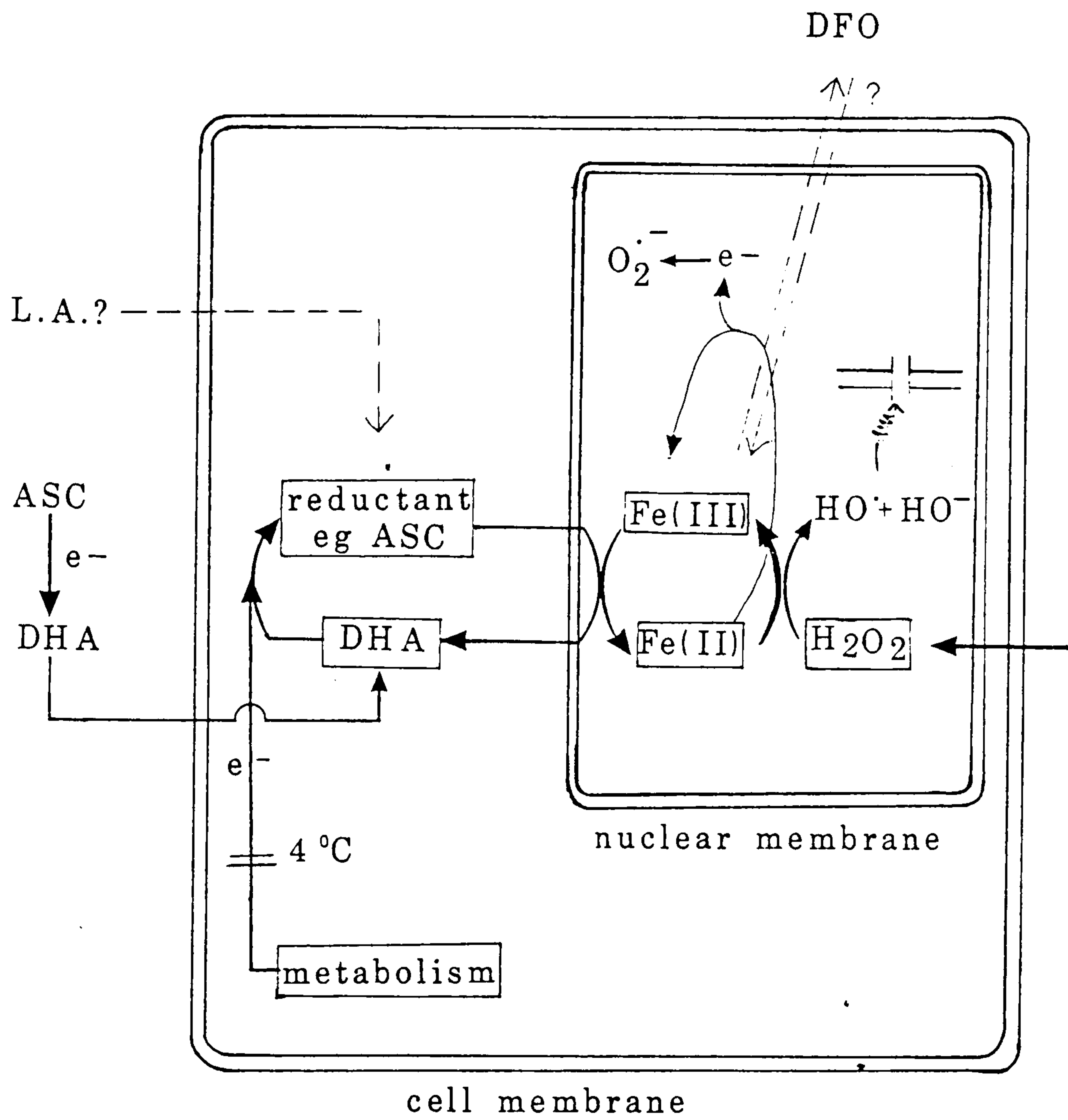


fig 4.30

temperatures below 15°C (Shirazi et al.,1982) consistent with findings by others (Pratten & Lloyd,1979). In a study by Damluji & Riley (1979) the effect of temperature on detachment of cells in the presence of various agents showed a transition effect between 18°C and 24°C, which was ascribed to a membrane lipid transition temperature of the cells.

Active transport mechanisms across cell membranes may be extended to sites within the cell which are continuous with the membrane. If the cytotoxic process of H_2O_2 involves the supply of reducing equivalents for iron to a particular part of the cell, then the transition temperature suggests that an active transport mechanism for the reduction is inhibited below this temperature. In this context the protective effect observed with zinc lends further support for the direct target being associated with the DNA. The location of specific sites in the DNA which are occupied by zinc have been recently reported for E.coli (Klug & Rhodes,1987). These "zinc fingers" are thought to lend structural support to the DNA and each zinc atom is liganded to 2 cysteine and 2 histidine residues. Of the transition metals iron is similar to zinc in ionic radius (see Table) and it would not be impossible for iron to substitute at certain sites for zinc.

ion	crystal radii Å
Fe^{2+}	0.76
Zn^{2+}	0.74
Fe^{3+}	0.64

(from Pauling,1960)

If reduced iron were to occupy some of these sites then in the presence of H_2O_2 the Fenton reaction could occur near the DNA. The cleavage of DNA by HO· radicals generated by iron and H_2O_2 has been utilized as a laboratory tool to study specific regions in detail (Tullis,1987). The cytotoxic action exerted by zinc aspartate and zinc histidine themselves limited the extent of any protective effect against H_2O_2 toxicity.

The inhibition of cell proliferation of mouse melanocytes _in vitro_ by high zinc concentrations has been studied by Borovanski & Riley (1983) but found to be ineffective _in vivo_ (Borovanski _et al_.,1985) possibly because the neccessary cytotoxic concentrations were not attained. In a recent study by Borovanski & Riley (1988) the addition of iron was shown to protect against the deleterious effects of zinc. An extensive review of zinc and iron in free radical pathology and cellular control has recently been given by Willson (1988).

4.4.3. Effects of iron complexes

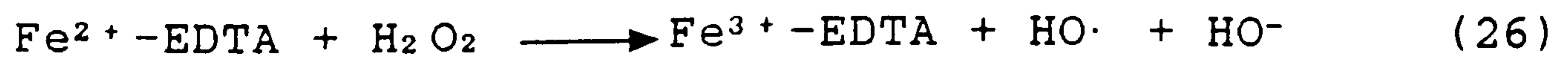
If the cytotoxicity of H_2O_2 is due to the Fenton reaction in the vicinity of the DNA it is questionable

whether this reaction occuring in any other part of the cell would result in similar damage. This was investigated by exposing cells to iron added extracellularly. This iron needed to be complexed and it is the nature of the ligand which determined the location of iron in the cell. From section 4.3.3 it is evident that the localisation of iron plays an important part in determining the effect of interaction with H_2O_2. Fe/EDTA (1:1) protects cells from H_2O_2 damage. In chapter III it was shown that cells which were incubated with Fe/EDTA and then lysed contained no additional ferrozine-detectable iron. Prussian blue staining was also negative. The half-life of H_2O_2 with Fe/EDTA suggests that an extracellular Fenton reaction may take place. The rate of autoxidation of Fe^{2+}/EDTA is more rapid than Fe^{2+}/H_2O as EDTA shifts the redox potential of Fe^{2+}/Fe^{3+} from -0.77V (Latimer,1952) to -0.12V (Schwarzenbach,1951). This favours the production of $O_2^{\cdot -}$:

$$Fe^{2+}(EDTA) + O_2 \longrightarrow Fe^{3+}(EDTA) + O_2^{\cdot -} \quad (25)$$

$k = 1 \times 10^{2}\ M^{-1}\ s^{-1}$ pH 7.6 (Bull,1983)

The rate constant for the oxidation of Fe^{2+}(EDTA) by H_2O_2 has been reported as $\sim 2 \times 10^{4}\ M^{-1}\ s^{-1}$ at pH 7.6 by 2 different authors (Bull,1983; Borggaard,1971).



$$Fe^{2+}\text{-EDTA} + H_2O_2 \longrightarrow Fe^{3+}\text{-EDTA} + HO\cdot + HO^- \quad (26)$$

Therefore, with a high concentration H_2O_2 and excess Fe/EDTA, most of the H_2O_2 will have been utilised extracellularly.

The presence of Fe^{3+}(EDTA) has also been shown to enhance HO· generation in a hypoxanthine/xanthine oxidase mixture at pH 7.4 as compared to $FeCl_3$ alone (Flitter et al.,1983). One reason that has been suggested for EDTA stimulation of the iron-catalysed Haber-Weiss reaction is that as a chelator EDTA retains the hydrolyzable Fe^{3+} in a soluble form (Tien et al.,1983; Baker & Gebicki,1984).

HO· radicals produced extracellularly do not damage the cell (Jacobs et al.,1985). Analogous arguments can be applied to other complexes which do not enter the cell. ATP and ADP both showed a negative enhancement factor and protected the cell from H_2O_2 damage. Evidence for the formation of HO· radicals in solutions of iron-nucleotides and H_2O_2 comes from studies using electron paramagnetic resonance DMPO spin trapping techniques (Floyd,1983). It was shown in these experiments that HO· radical formation in the presence of ADP or ATP was in the order of 20-50 fold higher than in the absence of the nucleotides. This was similarly achieved for Fe/EDTA (Floyd & Lewis,1983). Exposure of lenses in organ culture to H_2O_2 generated by xanthine/xanthine oxidase leads to damage of these lenses, which could be prevented by the

addition of Fe-nucleotide complexes (Zigler _et al_.,1985). The results described in this chapter are consistent with these findings.

Both Fe/8-HQ and Fe/dex were shown to enhance H_2O_2-induced cytotoxicity. The intracellular distribution following incubation with the 2 agents has been demonstrated. Fe/8-HQ exhibited a toxicity in its own right. In contrast to Fe/dex the intracellular distribution of Fe-8HQ was not associated with any particular compartment. Interaction with H_2O_2 would therefore be expected to result in a greater probability of inducing damage at critical sites within the cell. This is reflected by the high enhancement factor. In the case of Fe/dex, in which uptake of iron into vesicular organelles was demonstrated, damage induced by H_2O_2 may be secondary to reactions of HO· radicals within lysosomes. This could result in lysosomal membrane damage (eg initiation of lipid peroxidation) with subsequent release of hydrolytic enzymes. Generation of free radicals within the lysosome by photosensitisation has been shown to release hydrolases from the lysosome which could be potentially damaging (Slater & Riley, 1966).

When endocytosis is inhibited, ie. when cells were pre-exposed to Fe/dex at 4°C the enhanced cytotoxicity is absent. Inhibition of iron uptake was confirmed by the absence of Prussian blue staining and absence of any increase in ferrozine-detectable iron in cell lysates.

Some enhanced cytotoxicity was also seen when cells were pre-incubated with Fe/dex at 37°C, followed by exposure to H_2O_2 at 4°C. A possible explanation is that intracellular reduction of internalised Fe/dex occurs at 37°C and that at 4°C there is no barrier to the diffusion of H_2O_2 into the lysosomal compartment of the cell.

Zinc aspartate was shown to protect cells against the enhancement of the cytotoxicity of H_2O_2 by Fe/dex. In this case if the protection is brought about by interchange of zinc for iron it must be with iron that is associated with the vesicular compartment of the cell.

The results described may be summarised as follows:

1. The cytotoxicity of H_2O_2 involves the presence of transition metal ions at critical target sites in the cell. The primary damaging reaction is probably the Fenton reaction generating HO· radicals which damage important macromolecules (eg lipids, DNA).
2. Fe(II) complexes present extracellularly which are not internalised protect cells by reacting with H_2O_2 thereby reducing the concentration to which the cells are exposed. The degree of protection will be proportional to the quantity of Fe^{2+} in the complex at the time of H_2O_2 addition.
3. Fe(II)/complexes that are internalised by the cell will enhance the cytotoxicity of H_2O_2. The enhancement will be proportional to the amount of

iron and its location in the cell.

4. Fe(III)/complexes that are internalised by cells enhance cytotoxicity due to H_2O_2 if conditions exist for reduction of the iron. This effect will be proportional to the amount of iron, the degree of reduction and the intracellular location.

CHAPTER V: DIRECT INTERACTION OF FREE RADICALS WITH A LIGAND AND THE EFFECT ON YEAST ALCOHOL DEHYDROGENASE (YADH)

5.1 INTRODUCTION

In Chapter II the potential of ligands to influence the redox state of iron was discussed. Additional physico-chemical properties of the iron complex were taken into account with respect to discussing their effect on H_2O_2-induced cytotoxicity (Chapter IV). Attention is now focussed on the direct interaction of targeted radicals, which are generated by radiation, with different iron complexes or ligands.

The radicals investigated include the superoxide free radical, the thymine peroxy radical and the desferral nitroxide free radical.

Iron has been previously shown to protect YADH from various secondary radicals, in particular against the thymine peroxy radical and urate radical toxin (Mondon, 1985). The interaction of iron complexes with superoxide has also been extensively investigated (Halliwell & Gutteridge,1984). Many of these reactions are affected by the nature of the ligand to which iron is bound.

Yeast alcohol dehydrogenase (YADH) has been used as the biological endpoint. This enzyme has been extensively used in this laboratory to study the effects of primary and secondary radicals.

5.1.1 The structure and function of YADH

YADH is a tetramer with a molecular weight of about 150,000 (Bränden *et al*.,1975). Its structure has been deduced from peptide analysis (Jörnvall,1977), showing the presence of 347 unique residues in agreement with other authors (Harris,1964; Butler,thesis). The active yeast enzyme contains one (Veillon & Sytkowski,1975) or two (Klinman & Welsh,1976) zinc atoms per subunit. It is both a metallo- and sulphydryl enzyme and functions as a oxido-reductase (Sund & Theorell,1963).

YADH contains 8-9 SH groups per subunit, 2 of which are reactive sulphydryls (Bränden *et al*.,1975). The subunit which contains zinc and one reactive SH group binds both NAD^+ and NADH (Harris,1964). The other -SH groups are not involved with coenzyme binding (Sanderson & Weiner,1973). Each YADH molecule has 4 coenzyme molecules, each of which fits into a hydrophobic pocket, close to the zinc atom at the active site. The zinc atom is ligated by two cysteine residues and one histidine residue. This active site is separated from the coenzyme binding site by hydrophobic residues. In the presence of coenzyme, NAD^+, the enzyme undergoes a large conformational change from an open to a closed form, thus binding NAD^+ more tightly. This is followed by electrophilic catalysis, mediated by the zinc atom.
YADH catalyses the reaction of alcohol to aldehyde in the presence of coenzyme:

$$RCH_2OH + NAD^+ \longrightarrow RCHO + NADH + H^+ \quad (27)$$

In fact, in yeast cells the reverse reaction is common (the reaction of aldehyde to ethanol), however, both alkaline pH and the removal of aldehyde using excess semicarbazide ensures the *in vitro* direction to proceed as given above.

5.1.2 Radiation induced free radical generation

Studies of the effect of steady state radiolysis on the activity of various enzymes has been undertaken by various workers, eg. with lysozyme (Aldrich *et al*.,1969; Adams *et al*.,1969), alcohol dehydrogenase (Badiello *et al*.,1974; Land & Prutz,1979) and ribonuclease (Adams *et al*.,1971). The elucidation of pulse radiolysis data revealed the interaction of specific amino acids with radicals and it was suggested that some of these could be target residues on proteins. Such findings may be complicated, however, by the fact that the radical centre of some amino acids can be transferred to other residues in a peptide or protein (Prutz *et al*,1980 & 1981; Packer *et al*.,1981), eg.methionine radicals can interact with tryptophan and the radical from tryptophan can in turn interact with tyrosine and cysteine, (Prutz *et al*.,1982).

5.1.2.1 Formation of primary products from water radiolysis

Since the major constituent of living cells is water, exposure to ionising radiations will result in formation of the hydroxyl radical. Dilute solutions of biochemicals approximate the biological situation and thus it is water that absorbs nearly all the energy deposited.

The primary products form within about 10^{-9} seconds after initiation by the ionising ray, (Bielski & Gebicki,1977).

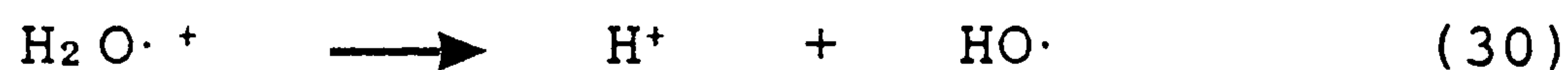
The first step involves ionisation of water:

$$H_2O \longrightarrow H_2O\cdot^{+} + e^{-} \qquad (28)$$

The electron hydrates at about 10^{-11} seconds:

$$e^{-} + nH_2O \longrightarrow e^{-}_{(aq)} \qquad (29)$$

Unstable water ion radicals decompose or undergo proton transfer:



$$H_2O\cdot^{+} \longrightarrow H^{+} + HO\cdot \qquad (30)$$

$$H_2O\cdot^{+} + H_2O \longrightarrow H_3O^{+} + HO\cdot \qquad (31)$$

A small but probably insignificant contribution to the overall radical yield is also made by excited water:

$$H_2O\cdot^* \longrightarrow H\cdot + HO\cdot \qquad (32)$$

Further reactions result in molecular products but with a smaller yield:

$$H\cdot + H\cdot \longrightarrow H_2 \qquad (33)$$

$$HO\cdot + HO\cdot \longrightarrow H_2O_2 \qquad (34)$$

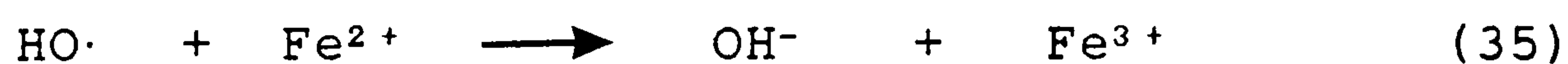
After about 10^{-9} seconds, $e^-_{(aq)}$, $H\cdot$, $HO\cdot$, H_2 and H_2O_2 begin to apear in bulk solution and are able to react with other solutes present.

5.1.2.2 Reactions of primary products

(i) Hydroxyl radical

In general the molecular products are unreactive. H_2O_2 is produced in such small yields by the average radiation doses applied that it is not considered to produce extensive oxidations.

The most common reactions concern the hydroxyl radical. Rections with Fe^{2+} (Electron transfer);



$$HO\cdot + Fe^{2+} \longrightarrow OH^- + Fe^{3+} \qquad (35)$$

or ethanol (hydrogen abstraction) or addition reactions, eg benzene are well documented (Bielski & Gebicki,1977)

(ii) Hydrated electron

The hydrated electron is of equal importance as a

reducing product. Many of its reactions have been studied and are summarized (Anbar et al.,1973). Nitrous oxide is an excellent scavenger of hydrated electrons ($k = 9.1 \times 10^9\ M^{-1}\ s^{-1}$, Janata & Schuler,1982). Thus 20% of the primary radicals are converted to the HO· radical;

$$e^{-}_{(aq)} + N_2O + H_2O \longrightarrow N_2 + OH^{-} + HO\cdot \qquad (36)$$

Oxygen is an effective scavenger of the primary reducing radicals. The rate constants for the reactions with H· and $e^{-}_{(aq)}$ are nearly $2 \times 10^{10}\ M^{-1}\ s^{-1}$ and since the concentration of O_2 in air saturated solutions is 2.5×10^{-4} M it competes effectively with most other solutes for these;

$$H\cdot + O_2 \longrightarrow HO_2\cdot \qquad (37)$$

$$e^{-}_{(aq)} + O_2 \longrightarrow O_2\cdot^{-} \qquad (38)$$

$HO_2\cdot$ and $O_2\cdot^{-}$ are related through the equilibrium

$$HO_2\cdot \rightleftharpoons O_2\cdot^{-} + H^{+} \qquad (39)$$

which has a pKa of 4.8 (Behar et al.,1970) or 4.7 (Bielski,1978).

Although the protonated form of superoxide is more reactive only about 1-2 % of $O_2\cdot^{-}$ will be in its protonated form at neutral pH.

(iii) Reactions of superoxide

It has been suggested that damage in cells could be caused by $O_2{\cdot}^-$ diffusing long distances from its site of production to sites of low pH, where it becomes protonated (Bielski,1983).

One of the most important reactions concerning superoxide involves the iron-catalysed decomposition of H_2O_2, commonly known as the Haber-Weiss reaction (reaction 9) and is described in Chapter I.

It must also be mentioned that the potential damage by $O_2{\cdot}^-$ in living organisms is prevented largely by the accelerated dismutation reaction by superoxide dismutase enzymes (Fridovich,1983; Halliwell,1983). This knowledge has been based on the fact that virtually all aerobic organisms possess S.O.D. activity, but in anaerobes these have not been found, suggesting their function to be a first line defence against $O_2{\cdot}^-$. Additionally compounds that can also donate H^+ ions as well as electrons, such as ascorbate and α-tocopherol also interact with $O_2{\cdot}^-$. The comparatively high rate constant for the oxidation of ascorbate by $O_2{\cdot}^-$ (2.7×10^5 M^{-1} s^{-1}, Goscin & Fridovich,1973) combined with the unusually high levels of ascorbate in living cells have been cited as one of the arguments against S.O.D. being essential for aerobic life, (Fee,1981).

5.1.2.3 Rections of secondary radicals

Provided sufficient scavenger of primary radicals is added a whole range of new radicals can be produced *in vitro*.

Of particular interest has been the addition of thymine which interacts with HO· to form the hydroxyl radical adducts, which then react with oxygen to form the peroxy radicals. This is a well established system (Willson,1970; Das *et al*.,1985; Fujita & Steenken,1981; Hazra & Steenken,1983). The sites of attack by the HO· radical have been extensively investigated, the preferential addition being to the C5 and C6 double bond. Pyrimidines and purines react with HO· and the solvated electron at almost diffusion controlled rates (k = $5x10^9 M^{-1}s^{-1}$ and $1.7x10^{10} M^{-1}s^{-1}$ respectively, from von Sonntag,1987) that of the H atom being about 2 orders of magnitude slower (k = $5x10^8 M^{-1}s^{-1}$, von Sonntag,1987). The radicals derived from pyrimidines add O_2 in aerated solutions and are converted to the corresponding peroxy radicals (Adams *et al*.,1969). Many of the products formed following attack on the 5 and 6 position are mutagenic (Adams,1972).

In other systems stable radicals can be formed and one of the common species referred to is the nitoxide radical, which due to its relatively long half-life can be studied by ESR. Examples of these include (i) interaction of radicals with the ESR trap DMPO (5,5-

dimethyl-1-pyrroline-N-oxide), (ii) the formation of methaemoglobin from haemoglobin resulting from aniline poisoning which involves a one-electron oxidation intermediate being the nitroxide (Heubner,1948) and recently desferrioxamine has shown to produce a nitroxide radical (Davies _et al_.,1987). The latter example is under investigation in this chapter.

Finally, the interaction of primary radicals with uric acid forms a long-lived stable but toxic species (Willson _et al_.,1985, Mondon,1985). This has not yet been identified.

In this chapter DFO is examined as an example of a direct interaction of free radicals with a ligand. The radicals are generated from water radiolysis. YADH is used to illustrate its subsequent potential damage. DFO B is one of the most widely clinically used chelator and is a powerful chelator of Fe(III) (Halliwell & Gutteridge, 1984). An account of its uses and potential side effects is given in chapter I.

5.2 MATERIALS AND METHODS

5.2.1 Gamma irradiation source

A facility for generating gamma rays is provided by a ^{60}Co source at Brunel. A plan of this is presented in fig. 5.1 The source has an activity of approximately 1000Ci (3.7×10^{13} Bq). Radioactive cobalt is wound in the centre of a metal plate from the outer cell on a steel cable. Surrounding the rod is a metal frame with holes suitable for placing test-tubes at chosen distances from the source. As the rod is not strictly a point source it is insufficient to apply the theoretical relationship of dose falling as the square root of the distance. Calculation of dosimetry by chemical means thus provides a more reliable estimation of the dose rate. Moreover, the half-life of the source is approximately 5.7 years which necessitates dosimetry measurements to be made periodically.

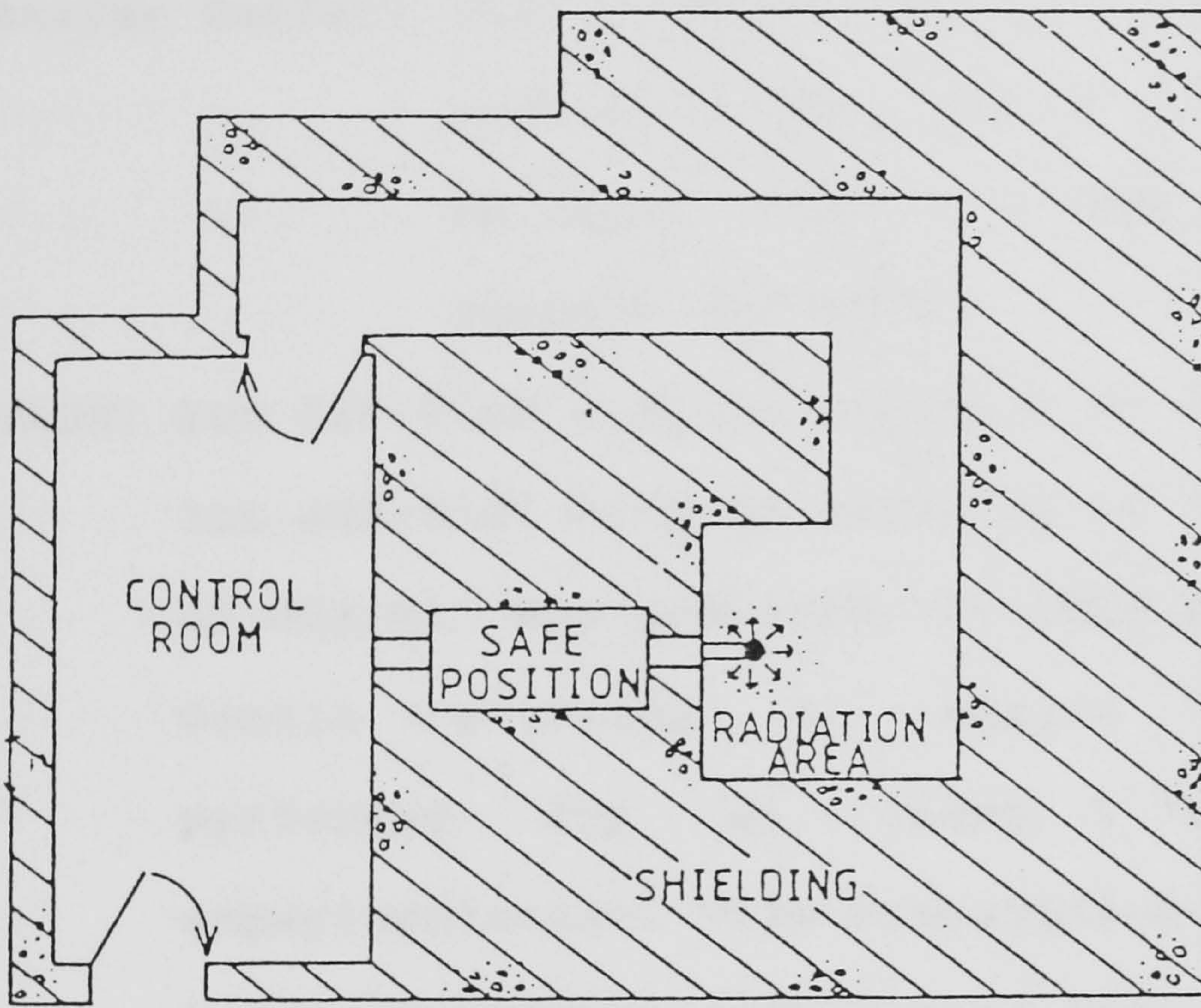
Details of steady-state radiolysis and dosimetry are given in Appendix V.

5.2.2 YADH Assay

The enzyme assay used to measure the remaining activity following radiation experiments was carried out according to a modified method of Gorin *et al.* (1967). The following solutions were prepared:

Phosphate buffer

Stock solutions of 20mM KH_2HPO_4 and K_2HPO_4 (Sigma) were prepared in double distilled water. Before any



Plan of the Brunel ^{60}Co gamma radiation facility.

fig 5.1

experiment both salts were mixed in proportions to give a pH of 6.7. For the substrate a solution of 12mM pyrophosphate (Sigma) was prepared in double distilled water and the pH adjusted to 8.5 with 1M HCl. The substrate contained nicotinamide adenine dinucleotide (NAD, Boehringer Co.Ltd.) EDTA, ethanol (BDH) and semicarbazide (hydrazinecarboxamide-HCl, Sigma).

Enzyme buffer: 20mM phosphate buffer. In any experiment involving the addition of desferrioxamine or other chelators YADH buffer did not contain any EDTA.

YADH: lot 84F-8080 with an activity of 290u/mg solid or lot 46F-8131 with an activity of 295u/mg. A stock of 80u/ml was prepared in 20mM phosphate buffer. Gentle stirring to ensure homogeneity was performed for at least 1 hour before any experimentation. This preparation was stable for 2-3 days without significant loss in activity.

Substrate: A solution of 12mM pyrophosphate buffer (pH 8.5) containing 1.25mM NAD^+, 0.11 absolute ethanol, 0.33mM EDTA and 73mM semicarbazide (which required neutralisation with 5M NaOH) was prepared.

The assay was carried out by addition of 0.02 mls of YADH solution to a 1 cm path length quartz cuvette containing 2.8mls of substrate. The solution was rapidly mixed with a plastic paddle and read against a substrate

blank at 340nm. The activity was assessed by the initial rate of NAD^+ to NADH. This produces a rate of change of optical density which is measured using a Perkin Elmer 3 spectrophotometer and R100 chart recorder. The assay was always carried out at 25°C. The initial slope is linear and the rate of NADH is measured over 30 seconds by the line drawn through the linear portion. The activity is expressed as a percentage of the unirradiated control activity.

5.2.3 Irradiation of all solutes present

10 ml solutions were prepared containing solutes of interest and a final enzyme concentration of 4u/ml. 3 mls were irradiated in thick walled test-tubes. The activity of YADH was measured immediately after irradiation, followed by measurement of the remaining unirradiated controls. In some cases solutions required the inclusion of a saturated gas. This was achieved by gently bubbling the gas through solutions for 10 minutes before sealing with parafilm. For Fe(II)-salts the solvent (water +/- ligand) was pre-gassed with N_2 followed by the addition of crystals of $FeSO_4$ under N_2.

Desferrioxamine at different concentrations was introduced into this system under conditions of both air saturation or N_2O/O_2 saturation. Similar experiments were carried out in the presence of excess formate (10^{-2}M) or in the presence of excess thymine (10^{-1} or 10^{-2}M). To these solutions the effect of addition of iron alone or

complexed to different complexes as well as the ligands themselves was studied.

5.3 RESULTS

5.3.1 Inactivation of YADH by radiation

The effects of irradiated solutions on the activity of YADH was studied. All systems contained the enzyme during irradiation. From previous studies damage to YADH has been principally attributed to the hydroxyl radical when no other solutes were present. Aerated solutions irradiated in the presence of only YADH resulted in a dose-dependent exponential inactivation (Gee,1986). The principal reactions responsible for damage to YADH can be summarized as follows:

$$HO\cdot + YADH \longrightarrow damage \quad (40)$$

$$e^-_{(aq)} + YADH \longrightarrow YADH\cdot^- \quad (41)$$

$$YADH\cdot^- + O_2 \longrightarrow O_2\cdot^- + YADH \quad (42)$$

also $$e^-_{(aq)} + O_2 \longrightarrow O_2.^- \quad (43)$$

$$O_2.^- + YADH \longrightarrow no\ damage \quad (44)$$

5.3.1.1 Reactions with superoxide ($O_2\cdot^-$) and desferrioxamine (DFO)

In the presence of excess formate (10^{-1} or 10^{-2}M) hydroxyl radicals are rapidly scavenged to give the carboxylate radical anion ($k = 3 \times 10^9\ M^{-1}\ s^{-1}$) (Hoe et al.,1982, Willson,1982);

$$HO\cdot + HCOO^- \longrightarrow CO_2\cdot^- + H_2O \quad (45)$$

which then reacts to produce superoxide, ($k = 4.5 \times 10^9$ M^{-1} s^{-1}, Bielski & Gebicki,1977)

$$CO_2^{\cdot -} + O_2 \longrightarrow O_2^{\cdot -} + CO_2 \quad (46)$$

Under conditions of N_2O/O_2 bubbled solutions all the hydrated electrons react to produce HO· resulting in twice the amount of $O_2^{\cdot -}$ being formed than under air;

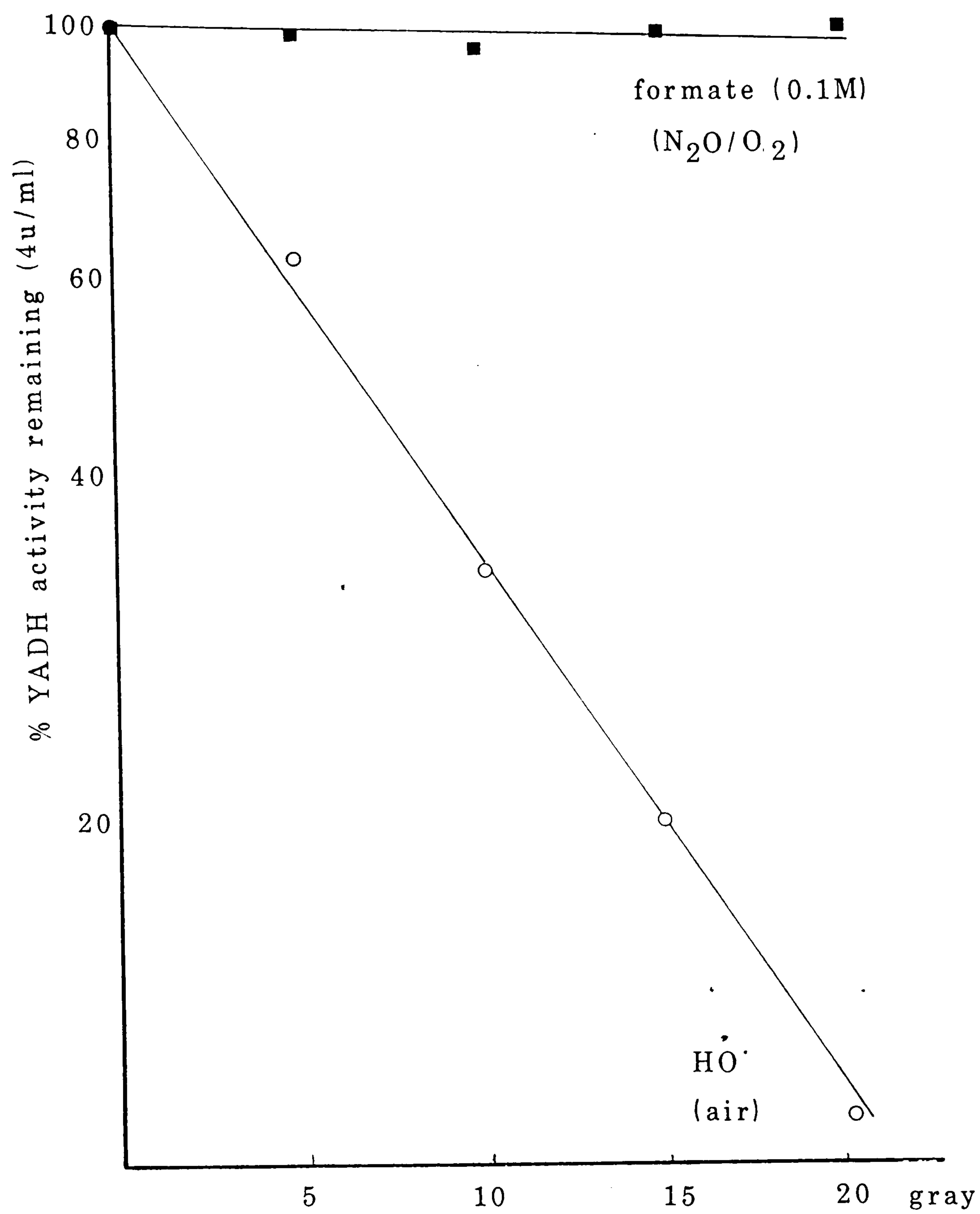
$$N_2O + e^-_{(aq)} \longrightarrow HO\cdot + N_2 + OH^- \quad (47)$$

$$HCOO^- + HO\cdot \longrightarrow CO_2^{\cdot -} + H_2O \quad (48)$$

$$CO_2^{\cdot -} + O_2 \longrightarrow O_2^{\cdot -} + CO_2 \quad (49)$$

The superoxide formed causes negligible damage to YADH (fig. 5.2). The role of iron under these conditions was investigated by the addition of desferrioxamine to the irradiated solutions. The possiblity of chelating out essential iron from the system may sensitize the enzyme to superoxide. A range of other ligands were tested (maximum 100µM) but only the presence of DFO proved considerable damage in the presence of excess formate, fig. 5.3. It was subsequently confirmed by ESR studies that desferrioxamine itself forms a stable nitroxide radical with a half life of approximately 10 minutes (Davies et al.,1987). The damage thus observed was attributed to this radical and not to the effect of chelating out iron from the system. Inactivation is dose-

PROTECTION OF YADH BY FORMATE



1mM phosphate buffer pH7

fig 5.2

INACTIVATION OF YADH BY DFO IN THE PRESENCE OF FORMATE

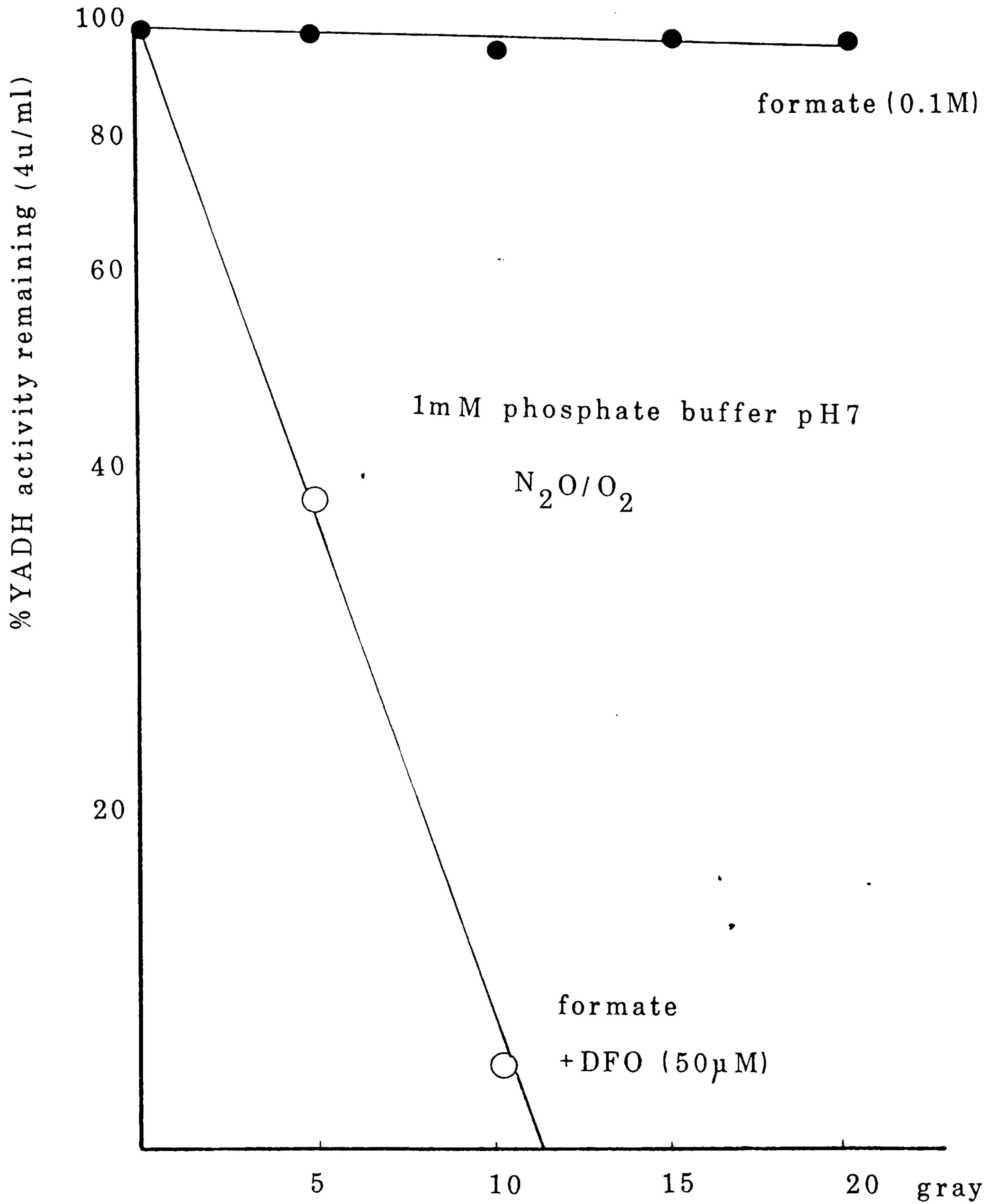


fig 5.3

dependent on DFO and is similar in both aerated and N_2O/O_2 bubbled solutions. The reactions thought to take place (Davies et al.,1987) in aerated solutions are:

$$e^-_{(aq)} + O_2 \longrightarrow O_2^{\cdot -} \quad (50)$$

or

$$e^-_{(aq)} + DfNO^- \longrightarrow DfNO^{\cdot 2-} \quad (51)$$

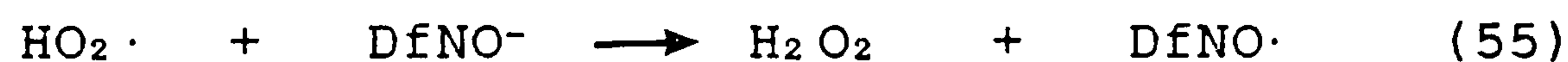
$$DfNO^{\cdot 2-} + O_2 \longrightarrow DfNO^- + O_2^{\cdot -} \quad (52)$$

With the presence of excess formate and twice the amount of $O_2^{\cdot -}$ being produced in N_2O/O_2 than in air, this anion can react further with $O_2^{\cdot -}$:

$$O_2^{\cdot -} + DfNO^- + 2H^+ \longrightarrow H_2O_2 + DfNO^{\cdot} \quad (53)$$

or:

$$O_2^{\cdot -} + H^+ \longrightarrow HO_2^{\cdot} \quad (54)$$



$$HO_2^{\cdot} + DfNO^- \longrightarrow H_2O_2 + DfNO^{\cdot} \quad (55)$$

followed by:

$$DfNO^{\cdot} + YADH \longrightarrow \text{inactivation} \quad (56)$$

If $FeSO_4$ (50μM) is added to the same solutions YADH is protected from damage by DFO provided YADH is assayed shortly after irradiation, (fig.5.4). Protection is also observed in the presence of mM methionine but not tryptophan, (fig. 5.5).

EFFECT OF DOSE OF DFO ON THE INACTIVATION OF YADH

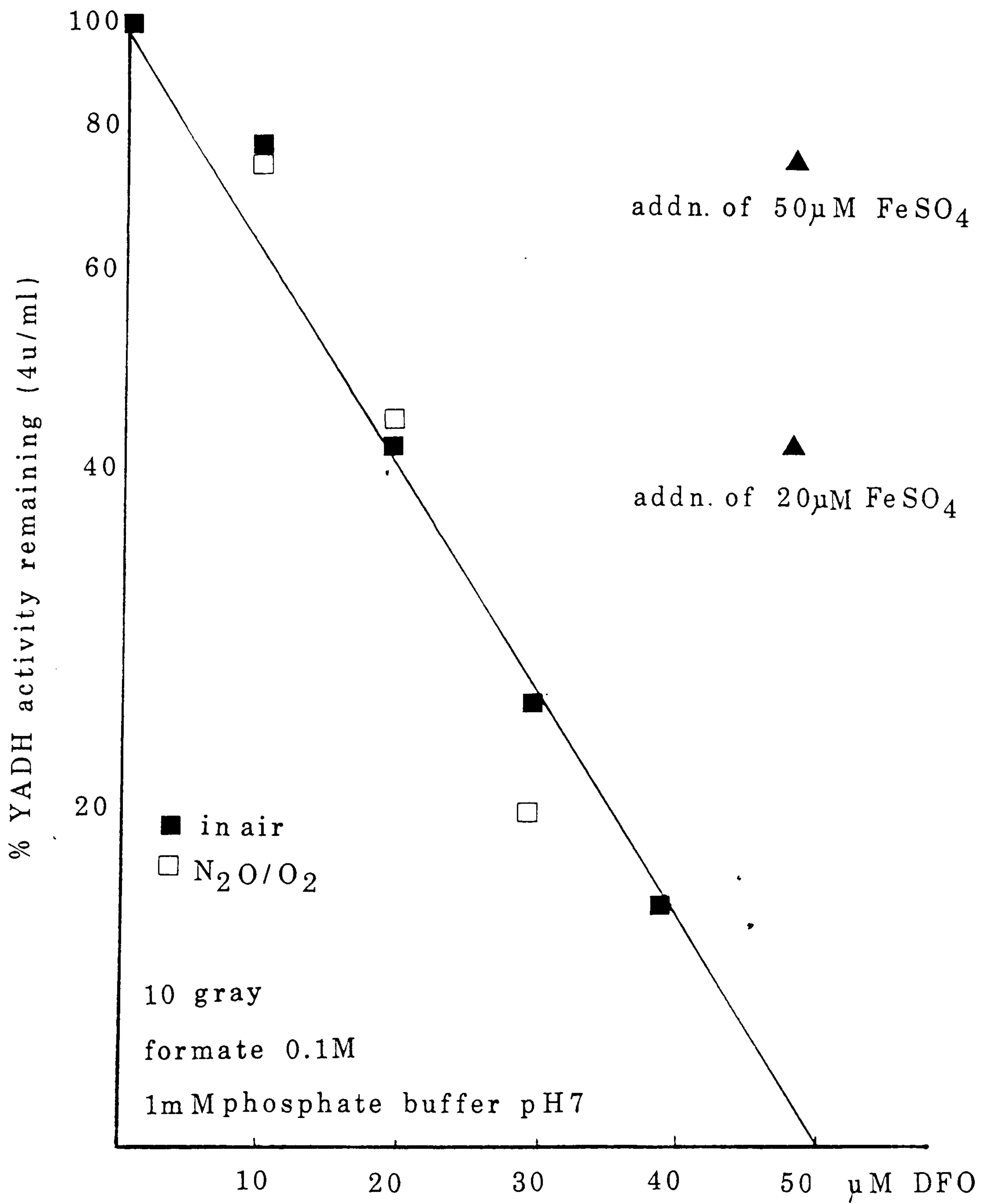


fig 5.4

EFFECT OF AMINO ACIDS ON THE INACTIVATION BY THE DFNO· RADICAL

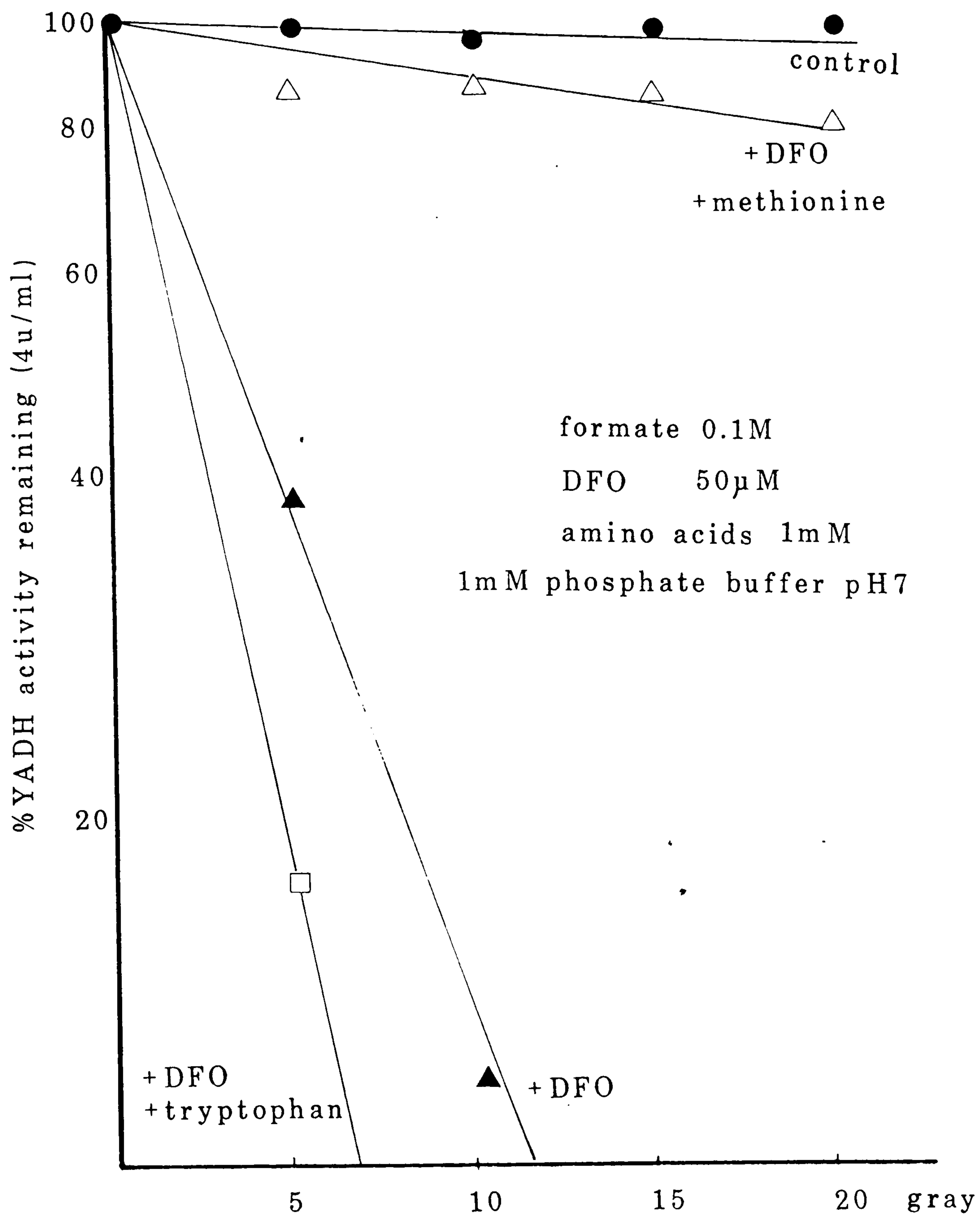


fig 5.5

Ascorbate (20 μM) added to the solution before irradiation was protective against inactivation by DfNO· (table 5.1).

Complete protection was also achieved if Fe/EDTA (less than 10 μM) was present at the time of irradiation. However, no such protection is seen with different batches of freshly openend Fe/dextrans (table 5.2). To achieve any significant protection with Fe/dextrans much higher concentrations are required than for Fe(III)/EDTA. Greater damage occurred if the irradiated solution containing the enzyme was left to stand for longer time intervals before assaying. This may account for the discrepancies which occurred in the final percentage inactivation by DFO from different experiments.

In the absence of formate DFO also causes considerable damage to YADH, but it can be completely protected by the addition of Fe(III)/EDTA (see table 5.2).

5.3.1.2 Effect of thymine peroxy radical on the activity of YADH

Inactivation of YADH by thymine peroxy radicals is well documented (Willson,1985).

Thymine reacts with HO· and the hydroxyl radical adduct reacts very rapidly with oxygen ($k = 2 \times 10^{9}\ M^{-1}\ s^{-1}$):

TABLE 5.1

PROTECTION OF YADH FROM THE NITROXIDE RADICAL BY ASCORBATE

Formate (mM)	DFO (μM)	Ascorbate (μM)	% YADH activity remaining
10	-	-	86
10	20	-	26
10	20	20	92

1mM phosphate buffer pH 7

4u/ml YADH, 4 Gray, N_2O/O_2

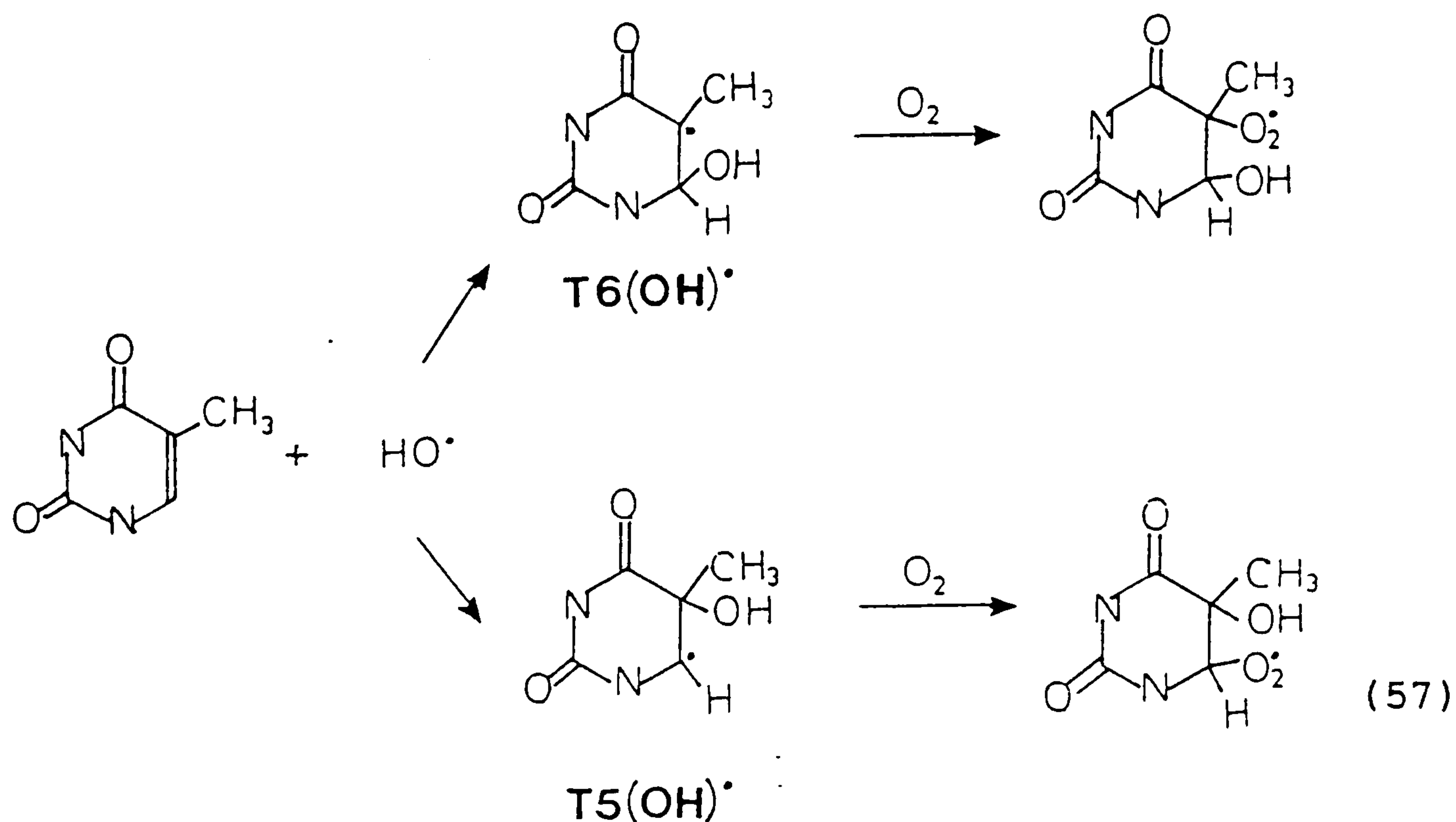
TABLE 5.2

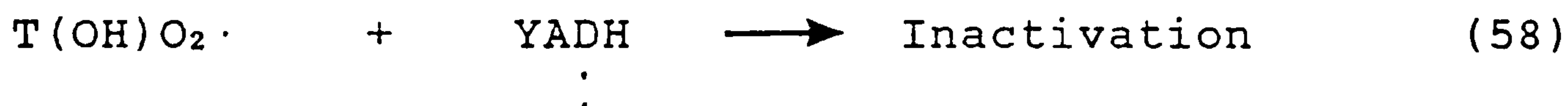
YADH INACTIVATION BY DFO AT THE TIME OF IRRADIATION IN THE PRESENCE OR ABSENCE OF FORMATE

Formate (mM)	DFO (μM)	Fe(III):EDTA 1 : 3 (μM) Fe	Fe/dex (μM) Fe	% YADH activity remaining
-	-	-	-	79
-	20	-	-	0
-	20	5	-	96
-	20	10	-	98
-	20	-	50	0
10	-	-	-	96
10	20	-	-	17
10	20	5	-	94
10	20	-	5	20
10	20	-	50	22
10	20	-	100	30

1mM phosphate buffer pH 7

4u/ml YADH, 4 Gray, in air





In an aerated solution containing 1mM thymine damage to YADH could be prevented by the addition of Fe(III)/EDTA and to some extent by Fe/dex (table 5.3). The addition of DFO to a thymine containing solution resulted in more inactivation of YADH than observed for the thymine peroxy radical alone (table 5.4). Fe(III)/EDTA also protected the enzyme in this system analogous to that found in the presence of formate (see table 5.2). Fe/dex showed very little protection; higher concentrations would be required to result in any appreciable protection.

TABLE 5.3

PROTECTION OF YADH FROM THE THYMINE PEROXY RADICAL BY Fe(III)/EDTA

Thymine (mM)	Fe(III)/EDTA 1:3 (μM) Fe	Fe/dex (μM) Fe	% YADH activity remaining
-	-	-	79
1	-	-	44
1	30	-	96
1	-	30	61
1	-	40	65

1mM phosphate buffer pH 7

4u/ml YADH, 4 Gray, in air

TABLE 5.4

THE EFFECT OF DFO ON YADH INACTIVATION BY THE THYMINE PEROXY RADICAL; PROTECTION BY Fe(III)/EDTA

DFO (μM)	Fe(III)/EDTA 1 : 3 (μM) Fe	Fe/dex (μM) Fe	% YADH activity remaining
-	-	-	44
20	-	-	4
20	30	-	98
20	-	30	6
20	-	40	20

1mM thymine

1mM phosphate buffer

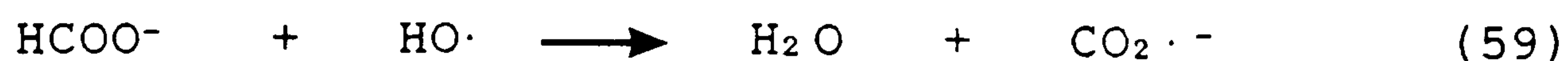
4u/ml YADH, 4 Gray, in air

5.3 DISCUSSION

1. The generation of free radicals was investigated using direct irradiation. The species principally concerned with the damage to YADH were (i) HO· from direct radiolysis of water, (ii) the product from interaction of the superoxide radical with DFO and (iii) the product from interaction of the thymine peroxy radical with DFO.

The addition of formate to irradiated solutions has been a useful tool to study conditions under which superoxide could potentially damage YADH. Under conditions of N_2O/O_2 bubbled or aerated solutions with only formate present superoxide does not damage (fig. 5.2). This is well established (Willson,1985; Gee *et al*.,1984; Halliwell & Gutteridge,1985).

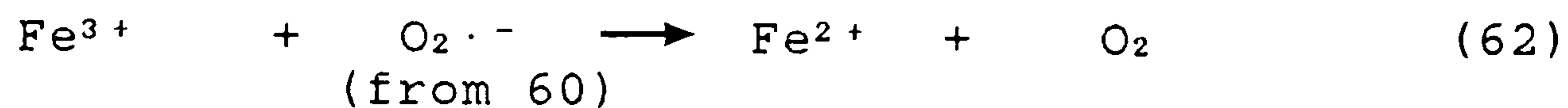
The effect of DFO in this system was at first surprising, as it was originally thought that this was due to its sensitizing effect to $O_2{\cdot}^-$. The protection by iron added as $FeSO_4$ directly to the irradiated solution could then have been associated with replacing iron which was removed by DFO. This iron present in the system may have artifactually protected YADH from $O_2{\cdot}^-$ damage. However, post-irradiation (enzyme addition after 10 mins) studies subsequently carried out in this laboratory showed that $FeSO_4$ does not protect against the DfNO· radical suggesting that ultimately $O_2{\cdot}^-$is reformed to react with DFO:



$$HCOO^- + HO\cdot \longrightarrow H_2O + CO_2\cdot^- \quad (59)$$

$$CO_2\cdot^- + O_2 \longrightarrow CO_2 + O_2\cdot^- \quad (60)$$

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2\cdot^- \quad (61)$$

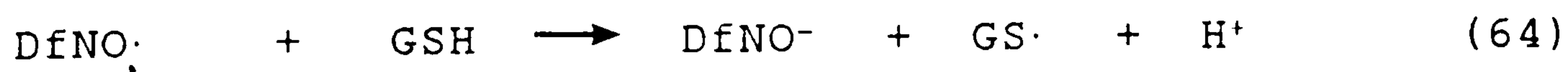


$$Fe^{3+} + \underset{\text{(from 60)}}{O_2\cdot^-} \longrightarrow Fe^{2+} + O_2 \quad (62)$$

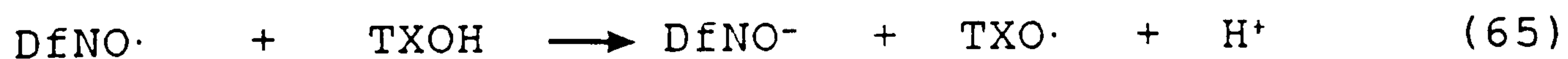
$$DFO + \underset{\text{(from 61)}}{O_2\cdot^-} \xrightarrow{2H^+} DFNO\cdot + H_2O_2 \quad (63)$$

The elucidation of the nitroxide radical by ESR followed post-irradiation studies carried out in this laboratory in which it was shown that this 'toxin' continued to be active long after irradiation.

Certain antioxidants which were included in the irradiated solution in studies subsequently undertaken proved to be effective against the inactivation by the nitroxide free radical. These included SOD, ascorbate,trolox C, cysteine, GSH and methionine (all 100μM) which were also effective if the enzyme was present at the time of irradiation, (Davies et al,1987). If antioxidants as well as YADH were added post-irradiation, then only cysteine, GSH and methionine were protective. The nitoxide ESR signal was absent in solutions containing GSH, trolox C and ascorbate implying the following reactions:



$$DfNO\cdot + GSH \longrightarrow DfNO^- + GS\cdot + H^+ \quad (64)$$



$$DfNO\cdot + TXOH \longrightarrow DfNO^- + TXO\cdot + H^+ \quad (65)$$

$$DfNO\cdot + VitCH \longrightarrow DfNO^- + VitC\cdot + H^+ \quad (66)$$

However, neither trolox C or vit C added post-irradiation were protective and this could be due to competitive reactions between the various products:

$$DfNO\cdot + enzyme \longrightarrow damage \quad (67)$$

$$DfNO\cdot + Vit\ CH \longrightarrow Vit\ C\cdot \quad (68)$$

$$DfNO\cdot + TXOH \longrightarrow TXO\cdot \quad (69)$$

where reaction (67) predominates over (68) and (69). In the irradiated solutions where antioxidant and enzyme are present at the time of irradiation the protection observed has been more likely attributed to competition with $O_2\cdot^-$ and the radicals formed from these reactions are not damaging to YADH:

$$O_2\cdot^- + Vit\ C \xrightarrow{H^+} Vit\ C\cdot + H_2O_2 \quad (70)$$

The protection observed with methionine may also be due to competition with $O_2\cdot^-$, although post-irradiation experiments showed that methionine added after irradiation also protected to some extent. The disappearance of the ESR signal would show whether methionine interacts directly with the nitroxide radical. Protection by methionine, if added post-irradiation aswell as enzyme could equally be due to interaction with DfNO· :

$$DfNO\cdot + MetSCH_3 \longrightarrow DfNO^- + MetS\cdot^+CH_3 + H^+ \quad (71)$$

The sensitivity of sulphydryl groups, eg. cysteine or methionine and indoles such as tryptophan to attack by oxidising free radicals is well known (Aldrich et al.,1969; Adams et al.;1972, Packer et al.,1981; Hiller et al.,1983). Methionine has been found to be particularly susceptible (Hiller & Asmus,1981, Hiller et al.1981).

Surprisingly, tryptophan did not protect if added during irradiation, implying little competition by tryptophan for $O_2^{\cdot -}$. It is remotely possible, however, that some reaction may also be occurring between DfNO· and tryptophan to give a species, which damages YADH more than DfNO· (see fig.5.5).

Of several iron complexes tested sofar Fe(III)/EDTA was shown to protect against the nitroxide radical if all solutes and enzyme were present at the time of irradiation.

Additionally, in the absence of DFO Fe(III)/EDTA was shown to protect against the thymine peroxy radical, (see table 5.5). This property of Fe/EDTA has not yet been fully investigated.

In the absence of formate when mainly HO· radicals are produced the damage is equally effective. The scavenging of HO· radicals directly is confirmed by pulse radiolysis studies from which the rate of HO· reaction

with DFO has been deduced as 5.3 x 10^9 $M^{-1}s^{-1}$ (Willson,1982). The protection by Fe/EDTA observed in the absence or presence of formate may be attributed to a direct interaction with the nitroxide radical, ie;

$$O_2{\cdot}^- + DFNOH \longrightarrow DFNO{\cdot} + H_2O_2 \qquad (72)$$

or

$$HO{\cdot} + DFNOH \longrightarrow DFNO{\cdot} + H_2O \qquad (73)$$

$$Fe^{3+}/EDTA + DFNO{\cdot} \longrightarrow DFNO^+ + Fe^{2+}/EDTA \qquad (74)$$

$$Fe^{2+}/EDTA + DFNO{\cdot} \xrightarrow{H^+} DFNOH + Fe^{3+}/EDTA \qquad (75)$$

overall:

$$2\ DFNO{\cdot} \xrightarrow{H^+} DFNOH + DFNO^+ \qquad (76)$$

This could be tested by post addition of Fe/EDTA aswell as enzyme to the irradiated solution. An alternative protective mechanism could also be operating in the presence of formate. Fe/EDTA can easily interact with superoxide (Halliwell, 1975). In the experiments carried out here the presence of $O_2{\cdot}^-$ would reduce the Fe^{3+} to Fe^{2+}. However, it may be that instead of a continuous redox cycle taking place, where the reduced iron autoxidises to produce more superoxide, Fe/EDTA could exert a dismutase effect. Some studies suggest that small coordination complexes of iron possess dimutase activity (Halliwell,1975; Pasternak & Halliwell,1979; Pasternak & Skowronek,1979; Peretz et al,1982). Pulse radiolysis

studies of iron-containing superoxide dismutases indicated a catalytic rate constant of $3 \times 10^{8}\ M^{-1}\ s^{-1}$ and suggested the intermediacy of $Fe-O_2{\cdot}^{-}$ complexes with the E.coli protein (Lavelli et al,1977; Fee,et al.,1981). The mechanism of Fe/EDTA catalysed superoxide dismutation has been studied by others (Bull et al.,1983) who have observed a rate constant for the reaction:

$$O_2{\cdot}^{-} + Fe^{3+}\text{-EDTA} \longrightarrow Fe^{2+}\text{-EDTA} + O_2 \qquad (77)$$

of about $2 \times 10^{6}\ M^{-1}\ s^{-1}$ with a pKa of 7.6.
Previous studies showed that $O_2{\cdot}^{-}$ would reduce Fe^{3+}-EDTA (Ilan & Czapski,1977) and participate in an oxidative addition reaction with Fe^{2+}-EDTA to form a peroxy complex (McClune et al.,1977)

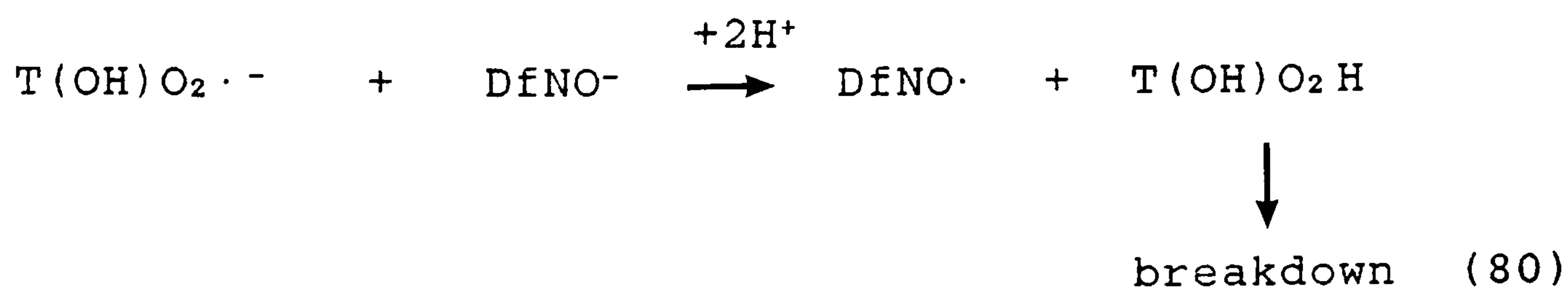
$$Fe^{2+}\text{-EDTA} + O_2{\cdot}^{-} \longrightarrow Fe^{3+}\text{-EDTA-}O_2{}^{2-} \qquad (78)$$

The direct observation of the peroxy complex during superoxide dismutation suggested that these reactions combine to form a catalytic cycle:

$$Fe^{3+}\text{-EDTA-}O_2{}^{2-} + 2H^{+} \longrightarrow Fe^{3+}\text{-EDTA} + H_2O_2. \qquad (79)$$

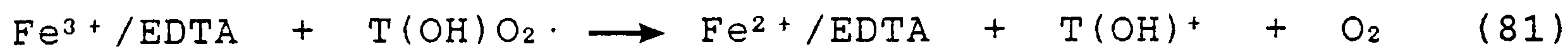
Evidence that DFO does not only damage YADH by interaction with superoxide was given by the reaction with the thymine peroxy radical to form a product with

greater potential to damage YADH. Post-effects have shown that the product also inactivates YADH after irradiation (this laboratory, unpublished results). The reaction could be:

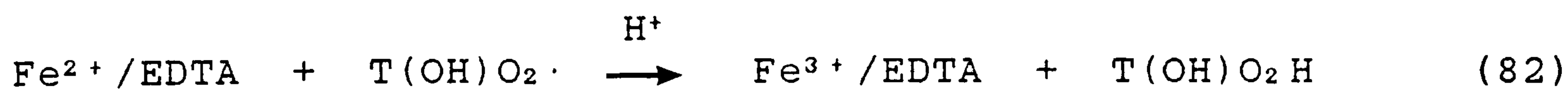


$$T(OH)O_2{\cdot}^- + DfNO^- \xrightarrow{+2H^+} DfNO{\cdot} + T(OH)O_2H \rightarrow \text{breakdown} \quad (80)$$

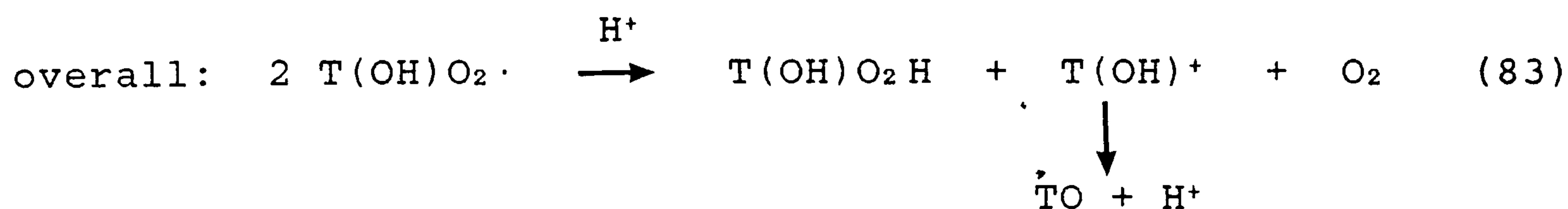
Fe/EDTA protected both against the thymine peroxy radical and the damage by the nitroxide radical, suggesting that protection occurs by preventing DFO from interacting with $T(OH)O_2{\cdot}^-$;



$$Fe^{3+}/EDTA + T(OH)O_2{\cdot} \longrightarrow Fe^{2+}/EDTA + T(OH)^+ + O_2 \quad (81)$$



$$Fe^{2+}/EDTA + T(OH)O_2{\cdot} \xrightarrow{H^+} Fe^{3+}/EDTA + T(OH)O_2H \quad (82)$$



$$\text{overall:} \quad 2\ T(OH)O_2{\cdot} \xrightarrow{H^+} T(OH)O_2H + T(OH)^+ + O_2 \quad (83)$$

$$T(OH)^+ \rightarrow TO + H^+$$

CHAPTER VI: GENERAL DISCUSSION AND CONCLUSIONS

It was the aim of this thesis to provide data supporting the suggestion that the Fenton Reaction is the predominant mechanism by which hydrogen peroxide damages cells *in vitro* and that the effect can be modified by the physico-chemical properties of iron complexes.

In the absence of any extracellularly added iron the toxicity of H_2O_2 was indirectly shown to be due to it interfering with the reductive metabolism of the cell. This was shown by altering the temperature of the exposure medium and adding external reductant which restored the toxicity of H_2O_2 at 4°C. The involvement of iron is implicated by the abolition of this effect by desferrioxamine (DFO). Whether or not DFO actually enters the cell is uncertain, but some evidence does exist that DFO can mobilise iron from the outside the cell. This is based on studies in which the incubation of cells with DFO resulted in an increase of measurable iron release into the extracellular medium (Mostert *et al.*,1986). If a reductive intracellular process is fundamental to the toxic effect by H_2O_2, then the provision of suitable reducing equivalents supplied from outside of the cell would have a potentially sensitizing effect. This could be a possible explanation for the observation that cells which have been exposed to small doses of radiation are sensitised to H_2O_2 (Alper,1961), ie if the production of hydrated electrons, hydrogen atoms or superoxide radicals

occurs near the vicinity of transition metals in the aqueous environment of the cell with the possibility of reduction then the potential for H_2O_2 damage is increased. Conversly, the partial protection against H_2O_2 observed in the presence of zinc salts is consistent with the finding that preloading mice with zinc salts protects these against high doses of radiation (Floersheim & Floersheim,1986). This supports the suggestion that sensitisation occurs as a consequence of redox cycling of transition metals in sensitive sites. Consistent with intracellular metabolic reduction of metal ions is the recent demonstration that exposure of E.coli to concentrations of H_2O_2 causes 'mode-one' type killing under normal metabolising conditions. However, starved cells could tolerate considerable exposure to H_2O_2 before they were killed, termed as 'mode-two' killing. This supports the notion that under these circumstances reductive metabolism is inhibited (Imaly et al., 1988).

The importance of the localisation of iron in affecting H_2O_2-induced cytotoxicity was demonstrated by the addition of iron complexes to cells. The distribution and redox state was shown to be a function of the ligand. Essentially 3 types of distribution were evident and these reulted in 3 different effects:

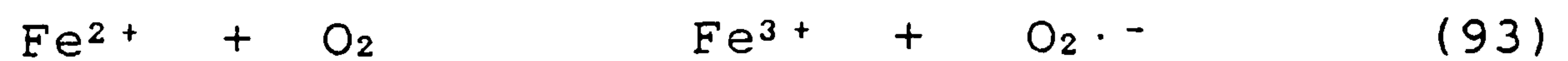
(i) Iron complexes which remained extracellular were protective against H_2O_2.

(ii) Intracellularly distributed iron complexes enhanced

H_2O_2 cytotoxicity.

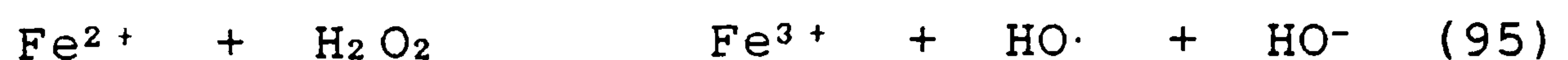
(iii) Iron associated with the vesicular compartment of cells enhanced H_2O_2 cytotoxicity.

Extracelluar Fe(II) complexes exhibited a limited toxic effect in their own right. This was observed for Fe(II)/EDTA, Fe(II)/phen (demonstrated in Chapter IV) and Fe(II)/ATP (demonstrated in Chapter III). However, Fe(III)/complexes were not damaging. In the extracellular medium (ie PBSi) the rate of autoxidation of certain Fe(II)/complexes were shown to be rapid (e.g. Fe(II)/ATP and Fe(II)/EDTA)). The sequence of reactions occurring leading to the production of oxidised iron and water via a sequence of one electron oxidations (Cher & Davidson, 1955; Goto et al.,1970) are:



$$Fe^{2+} + O_2 \quad Fe^{3+} + O_2^{\cdot -} \qquad (93)$$

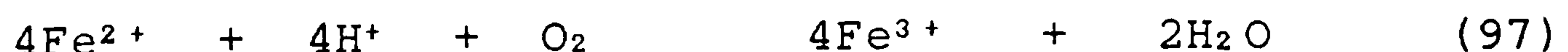
$$Fe^{2+} + 2H^+ + O_2^{\cdot -} \quad H_2O_2 + Fe^{3+} \qquad (94)$$



$$Fe^{2+} + H_2O_2 \quad Fe^{3+} + HO\cdot + HO^- \qquad (95)$$

$$Fe^{2+} + HO\cdot \quad Fe^{3+} + H_2O \qquad (96)$$

overall:



$$4Fe^{2+} + 4H^+ + O_2 \quad 4Fe^{3+} + 2H_2O \qquad (97)$$

Thus a small amount of H_2O_2 can be produced extracelluarly. However, this will react preferentially with extracellular Fe(II) instead of diffusing through the plàsma membrane as was demonstrated by the protective

effect in the presence of H_2O_2 and extracellular Fe(II). The effect observed with Fe(II)/complexes alone may therefore be due to direct interaction with components on the plasma membrane. These could be labile thiol groups, eg. cysteine, or other amino acids such as histidine. Extensive evidence exists for the potential damage caused by interaction of SH groups with iron in the presence of oxygen with studies dating back to the last century (Loven,1884; Bauman,1884; Mathews & Walker,1909; Lanform & Nielsen,1957; Wills & Wilkinson,1967; Misra,1974). Identification of hydroxyl free radicals by ESR in the presence of solutions containing cysteine and iron could account for some of the potential damage (Searle & Tomasi,1982).

Fe/8-hydroxyquinoline (HQ) which was internalised by the cell was also shown to be toxic by itself. If the extracellular damage can be associated with thiol interaction then this possibility may be extended to intracellular damage observed with Fe/8-HQ. In view of the suggestions made concerning 'decompartmentalised' iron (Willson,1977), both the physical and distance related barriers have been exceeded by this complex, making interaction with reactive molecules a realistic possibility. In 1973 Albert illustrated this with what he termed the 'partition effect', ie the transport of a metal by a chemical into compartments, with his studies on 8-HQ. This effect is

secondary to the enhanced toxicity observed in the presence of H_2O_2.

Fe/dex was associated with the vesicular compartment of the cell. Vesicles are topologically extracellular. However, vesicular membranes are structurally and biochemically different from the plasma membrane. For instance, the lysosome contains special transport proteins that use ATP to pump H^+ into the lumen of the lysosome, thereby maintaining the pH of the lumen at 5 (Alberts et al.,1985). Following any diffusion of H_2O_2 across the lysosmal membrane, the damage associated with endocytic vesicles containing Fe/dex are thus secondary to the damage caused by H_2O_2 itself.

Both these secondary effects induced by H_2O_2 support the suggestion that multiple sites in the cell can be potential targets for damage. The toxicity of H_2O_2 itself is well documented (Rhaesse & Freese,1968; Massie et al., 1972; Welsh et al., 1985) but the mechanisms of cell injury are still controversial. The most popular view is that of direct damage to DNA but the data presented here suggest that this need not be the only target.

The secondary effects described can be of considerable importance in the context of the observed exacerbation of rheumatoid arthritis following Fe/dex treatment. In 1969 5 out of 7 patients were reported to have suffered from exacerbations of joint pain (Reddy &

Lewis,1969). Some have ascribed this effect to a delayed hypersensitivity reaction (Lloyd & Williams, 1970) but others have suggested this to be due to a direct effect of lipid peroxidation in the synovial membrane (Blake et al.,1985). H_2O_2 generated by phagocytes could easily penetrate into the synovial cells and react with iron mobilised from ferritin as well as with Fe/dex from infusions (Halliwell et al,1985).

So far consideration was given only to the effect of a ligand on the reactivity of iron. However, at least of equal importance is the potential for interaction of free radicals with the ligand molecule directly. This was suggested to be the mechanism responsible for the rapid increase in O_2 utilisation by Fe/complexes in the presence of ascorbate and H_2O_2. The direct interaction of free radicals with the ligand was shown using DFO (Chapter V) as an example to illustrate the potential damage to a ligand itself.

The fact that DFO forms a relatively stable nitroxide radical is important in relation to some disturbing side-effects reported, such as visual and neurotoxicities (Simon et al.,1983; Arden et al.,1984; Olivierie et al.,1986) or those associated with its administration to patients with rheumatoid arthritis (Blake et al.,1985). In relation to cancer too there is evidence for the formation of nitroxide free radicals during' the metabolism of some carcinogens (Floyd et

al.,1967; Stier *et al.*,1972; Fisher and Mason,1986).

Although a catalytic effect of Fe/DFO in the presence of H_2O_2 and ascorbate was not evident (see fig 2.14 Chapter II), an attack on the ligand is nevertheless feasible and it would be interesting to examine the product by ESR in this particular system.

Clearly, further studies are required to establish whether or not a Fenton reaction is responsible for the observed potentiation of the cytotoxic effects of H_2O_2 in the presence of iron complexes. Of particular interest in this respect are the results concerning the endocytosis of iron dextran ,which may be relevant to some of the observed side effects when used therapeutically.

APPENDIX I

Student's T-test

The deviation from the mean is given by the standard deviation (SD) and is calculated by:

$$SD = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$$

where x = the individual observations , $\bar{x}$ = the mean of the observations and n= number of observations. The significance of differences from the mean was tested using the Student's' t-test, where for two sample populations:

$$T = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{SD^2}{n_1} + \frac{SD^2}{n_2}}}$$

APPENDIX II

Linear regession analysis

This calculates the slope and the y intercept for the best line. The line must fit the equation in the form of

$$Y = a + bx$$

and minimizes the pattern of vertical y deviations (prediction errors). The sum of all the deviations Σd is minimized:

$$\Sigma d^2 = \Sigma(y - Y)^2$$

From this the slope is calculated as:

$$b = \frac{\Sigma(x-\bar{x})(y-\bar{y})}{\Sigma(x-\bar{x})^2}$$

and shows how variables are linearly related. The degree to which they are related is given by the correlation coefficeint and is calculated from:

$$r = \frac{\Sigma(x-\bar{x})(y-\bar{y})}{\sqrt{\Sigma(x-\bar{x})^2 \; \Sigma(y-\bar{y})^2}}$$

```
READY.

 10 REM REGRESSION PRINTER
 20 POKE59468,14
 30 PRINT"❐
 40 PRINT" REGRESSION & CORRELATI■■■":
 80 PRINT:PRINT:PRINT
 90 POKE33728,18:POKE33729,21:POKE33730,14
 95 POKE371,49:POKE372,49
 100 POKE33733,48:END
 110 Z=33728
 120 READXL,YL:RESTORE
 130 READX$,Y
 140 X=VAL(X$)
 150 IFX$="END"THEN270
 160 N=N+1
 170 IFXL>XTHENXL=X
 180 IFXH<XTHENXH=X
 190 IFYL>YTHENYL=Y
 200 IFYH<YTHENYH=Y
 210 X1=X1+X
 220 X2=X2+X*X
 230 Y1=Y1+Y
 240 Y2=Y2+Y*Y
 250 XY=XY+X*Y
 260 GOTO130
 270 CV=N*XY-(X1*Y1)
 280 VX=N*X2-(X1*X1)
 290 VY=N*Y2-(Y1*Y1)
 300 R=CV/SQR(VX*VY)
 310 R5=INT(R*100)/100
 320 B1=CV/VX
 330 B5=INT(B1*100)/100
 340 C1=(Y1-B1*X1)/N
 350 C5=INT(C1*100)/100
 360 B2=CV/VY
 370 B6=INT(B2*100)/100
 380 VY=N*Y2-(Y1*Y1)
 385 C2=(X1-B1*Y1)/N
 390 C6=INT(C2*100)/100
 400 IFC6<0THEN420
 440 GETQ$:IFQ$=""THEN440
 450 IFQ$="Y"THEN500
 470 IFC6<0THEN490
 480 PRINT"X'=";B6;"Y";" + ";C6:GOTO540
 490 PRINT"X'=";B6;"Y";" ";C6:GOTO540
 510 IFC5<0THEN530
 520 PRINT"Y'=";B5;"X";" + ";C5:GOTO540
 530 PRINT"Y'=";B5;"X";" ";C5
 550 T=INT(100*R*SQR((N-2)/(1-R*R)))/100
 570 FORI=0TO19:POKEZ-I*40,93:NEXT
 580 FORI=0TO39
 590 POKEZ+I,64
```

```
600 NEXT
610 XR=XH-XL
620 S$=STR$(XL)
630 FORI=1TOLEN(S$)
640 XS=VAL(MID$(S$,I,1))
650 POKEZ+I-1,XS+48
660 NEXT
670 T$=STR$(XH)
680 FORI=LEN(T$)TO1STEP-1
690 XT=VAL(MID$(T$,I,1))
700 POKEZ+I-LEN(T$)+39,XT+48
710 NEXT
720 YR=YH-YL
730 RESTORE
740 U$=STR$(YL)
750 FORI=1TOLEN(U$)
760 YS=VAL(MID$(U$,I,1))
770 POKEZ+I-41,YS+48
780 NEXT
790 FORI=1TON
800 READX,Y
810 XT=INT((X-XL)/XR*40)
820 YT=INT((YL-Y)/YR*20)
830 POKEZ+XT-1+40*YT,46
840 NEXT
850 XM=INT((X1/N-XL)/XR*40)
860 YM=INT((YL-(Y1/N))/YR*20)
870 POKEZ+XM-2+40*YM,13
880 V$=STR$(YH)
890 FORI=1TOLEN(V$)
900 YW=VAL(MID$(V$,I,1))
910 POKEZ+I-801,YW+48
920 NEXT
930 POKEZ-360,25
940 POKEZ-320,1:POKEZ-319,24:POKEZ-318,9
945 POKEZ-317,19
950 POKEZ+10,24:POKEZ+12,1:POKEZ+13,24
955 POKEZ+14,9:POKEZ+15,19
960 POKEZ-20*40,93:POKEZ,109:POKEZ-40,93
970 IFXL<0THENPOKEZ,45
980 IFYL<0THENPOKEZ-40,45
2500 OPEN1,4
2510 FORII=0TO24:PRINT#1,:FORJJ=0TO39
2520 QQ=32768+JJ+40*II
2530 XX=PEEK(QQ):UU=XX+64
2540 IFXX>26THENUU=XX
2550 PRINT#1,TAB(1)CHR$(UU);
2560 NEXT:NEXT
3000 END
```

APPENDIX III

Calculation of degradation rate (k) of H_2O_2

$$[C_t] = [C_o].\ e^{-kt}$$

where C_o = initial concentration of H_2O_2

C_t = concentration of H_2O_2 at time t

t = time in minutes

k = decomposition rate of H_2O_2 at a particular cell density

Hence,

$$k = \frac{\log_e [C_t]}{\log_e [C_o].\ t}$$

APPENDIX IV

Calculation of total dose (D) in µM mins

$$D_{0-T} = \int_{t=0}^{t=60} (C_0 \; e^{-kT})\,dt$$

which gives:

$$D_{0-T} = \frac{C_0}{k} (1 - e^{-kT})$$

where D_{0-T} = total dose

and T = 60 minutes.

```
READY.

 100 REM DOSIMETER
 110 REM SONJA @ 9-MAY-88
 120 REM CALCULATES TOTAL DOSE FROM INITIAL CONC.
 122 REM CELL NUMBER & EXPOSURE TIME
 125 INPUT"SCREEN (3) OR PRINTER (4)";U:OPEN1,U
 130 AL=0.0038:REM  INTERCEPT VALUE FOR RATE OF H2O2 DECOMP
 140 BE=2.31E-9:REM SLOPE OF LIN REG OF DECOMP VS CELLS
 150 INPUT"EXPOSURE TIME (MINS) ",T
 160 INPUT"INITIAL CONCENTRATION (MICROMOLAR) ",C0
 170 N=0:D=0
 180 INPUT"CELL NUMBER (*10↑5)";N
 190 REM CALCULATION
 200 K=AL+BE*N*1E5
 210 D=(C0/K)*(1-EXP(-K*T))
 220 PRINT#1,"EXP TIME:"T"MINS","INIT CONC:"C0"MICROMOLAR",
 222 PRINT#1,"CELL NO:"N"*10↑5"
 225 PRINT#1,"DECOMP RATE:"K
 230 PRINT#1,"TOTAL DOSE ="INT(D)" MICROMOL.MIN."
 240 PRI?T#1,
 250 GOTO170
 260 CLOSE1:END
READY.
```

APPENDIX V

Steady-state radiolysis and dosimetry

The products of radiolysis of water yield a certain number of radicals, which is referred to as the G value. The G value is defined as the number of molecules or radicals formed or destroyed per 1.062×10^{-17} J (100eV) of radiation energy absorbed. Pure water yield G values of:

H_2O	$e^-(aq)$	$HO\cdot$	$H\cdot$	H_2	H_2O_2	H_3O^+
G	2.7	2.7	0.5	0.45	0.7	2.7

for gamma rays. The dose of radiation is expressed in terms of gray (formerly rad), where 1 gray = 100 rad, and its units are specified in Jkg^{-1}. In the case of water it is JL^{-1}. When calculating the number of radicals produced per litre of water the G value is approximately 6 (ie. $HO\cdot$, e^-, $H\cdot$) and the concentration of radicals would be

$$\frac{1(Jkg^{-1}) \times 6}{1.602 \times 10^{-17}(eV)} \times \text{Dose (Gy) per L.}$$

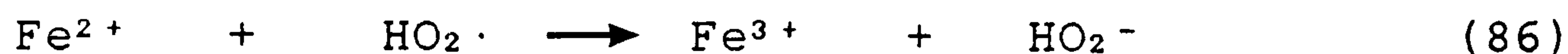
Thus for 1 Gray the number of radicals is 3.74×10^{17} or $(\div 6.023 \times 10^{23}) = 0.6$ μM.

Dosimetry is routinely carried out using Fricke dosimeter (Fricke & Hart, 1966). It consisits of a saturated solution of 1mM $FeSO_4$, 0.8N H_2SO_4 and 1mM NaCl (which

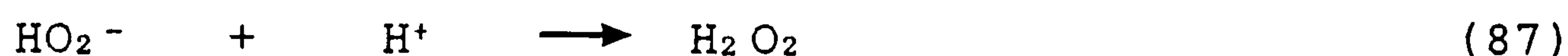
desensitizes the solution against organic impurities). Radiation induces the oxidation of $FeSO_4$ to $Fe(SO_4)_3$ which is detactable at 304nm. The vessels and volumes used are the same as used for radiation experiments. The following reactions occur in this solution:

$$Fe^{2+} + HO\cdot \longrightarrow Fe^{3+} \qquad (84)$$

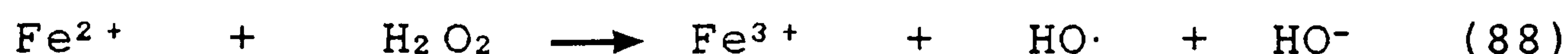
$$H\cdot + O_2 \longrightarrow HO_2\cdot \qquad (85)$$



$$Fe^{2+} + HO_2\cdot \longrightarrow Fe^{3+} + HO_2^- \qquad (86)$$



$$HO_2^- + H^+ \longrightarrow H_2O_2 \qquad (87)$$



$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO\cdot + HO^- \qquad (88)$$

The total yield of Fe^{3+} is given by:

$$G(Fe^{3+}) = 2G(H_2O_2) + 3G(H\cdot) + G(HO\cdot)$$

Fricke (1966) reports the following yield of G values for aerated solutions of ferrous sulphate:

$G(H\cdot)$	$G(HO\cdot)$	$G(H_2)$	$G(H_2O)$	$G(e^-_{(aq)})$
3.7	2.92	0.39	0.78	0

total $G(Fe^{3+}) = 15.6$

and the dose in gray is given by:

$$D(Gy) = \frac{6.023 \times 10^{23} \times OD_{304} \times 100}{E_{304} \times 10^3 \times G(Fe^{3+}) \times f \times p \times 1}$$

where 6.023×10^{23} = Avogadros number

OD_{304} = change in OD between irradiated solution and unirradiated control

E_{304}	= difference in molar extinction coefficient of Fe^{2+} and Fe^{3+} at 304nm
$G(Fe^{3+})$	= 15.6
p	= density of irradiated solution
	= 1.024 for 0.8N H_2SO_4
l	= optical path length
f	= 6.24×10^{11} eV/Gy

REFERENCES

Aasa,R., Malström,B.G., Saltman,P., Vänngard,T. (1963) Biochim Biophys Acta 75, 203

Adams,G.E. (1987) Br J Cancer 55, 11

Adams,G.E. (1972) in: "Advances in Radiation Chemistry" 3, 125 (eds M.Burton & J.L.Magee)

Adams,G.E., Aldrich,J.E., Bisby,R.H., Cundall,R.B., Redpath,J.L., Willson,R.L. (1972) Radiat Res 49, 278

Adams,G.E., Willson,R.L., Aldrich,J.E., Cundall,R.B. (1969) Int J Radiat Biol 16, 333

Adams,G.E., Willson,R.L., Bisby,R.H., Cundall,R.B. (1971) Int Radiat Biol 20, 405

Adeniyi,W. & Jaselskis,B. (1980) Talanta 27, 993

Aisen,P. & Listowski,I. (1980) Ann Rev Biochem 49, 357

Aisen,P. (1977) in: "Iron Metabolism" 1-15 Ciba Foundation Symposium

Aisen,P. & Leibmann,A. (1968) Biochim Biophys Acta 304, 797

Albert,A. (1953) Biochem J 54, 646

Albert,A. (1973) in: "Selective Toxicity" 5th ed Chapman & Hall

Albert,A. (1986) in: "Selective Toxicity" , 431-488 7th ed Chapman & Hall

Alberts,B., Bray,D., Lewis,J., raff,M., Roberts,K., Watson, J.D. (1985) in: "Molecular Biology of the Cell" p.367 Garland Publishing Inc

Aldrich,J.E., Cundall,R.B., Adams,G.E., Willson,R.L. (1969) Nature 225, 632

Allen,A.O., Hochanadel,C.J., Ghormley,J.A., Davies,T.W. (1952) J Phys Chem 56, 575

Alper,T. (1987) Br J Cancer 55, 32

Alper,T. (1961) in: "Mechanisms in Radiobiology" (ed. M.Emera & Forssberg) 1, p.353 Academic Press N.Y.

Anbar,M., Bambenek,M., Ross,A.B. (1973) Ref Data Ser, Nat Bur Stand 43, 23

Anusiem,A.C.I. & Gbeminiyi,BO. (1978) Anal Chem 40, 531

Arden,G.B., Wonke,B., Kennedy,C., Huehns,E.R. (1984)
Br J Opthalmol 68, 873

Arrick,B.A., Nathan,C.F., Griffith,O.W., Cohn,Z.A. (1982)
J Biol Chem 257, 1231

Astania,A.N. & Rudenko,A.P. (1971) Zh Fiz Khim 45, 352

Astania,A.N., Rudenko,A.P., Kuznetsova,N.A. (1972)
Zh Fiz Khim 46, 369

Aust,S.D., Morehouse,L.A., Thomas,C.E. (1985)
J Free Rad in Biol & Med 1, 3

Awai,M. & Brown,E.B. (1963) J Lab Clin Med 61, 636

Bacon,B.R., Tavill,A.S., Brittenham,G.M. Park,C.H., Recknagel,R.O. (1983) J Clin Invest 71, 429

Baker,M.S. & Gebicki,J.M. (1984)
Arch Biochem Biophys 234, 258

Bainton,D.F. & Finch,C.A. (1964) Am J Med 37, 62

Bauman,E. (1884) Z Physiol Chem 282

Barankiewicz,J. & Cohen,A. (1987)
Biochem Pharmac 36, 2243

Barb,W.G., Baxendale,J.H., George,P., Hargrave,K.R. (1951) Trans Faraday Soc 47, 462

Behar,D., Czapski, Rabini,L., Dorfman,L.M., Schwarz,H.A. (1970) J Phys Chem 74, 3209

Beinert,H. (1978) in: " Methods in enzymology" 54, p 435-445 (eds S.Fleisher & L.Packer) Academic Press N.Y.

Berger,N.A. & Berger,S.J. (1988)
Conference Abstract: "Biological Membranes in cancer Cells" June 13-16

Bernat,I. (1983) in: "Iron Metabolism" Plenum Publ Corp

Beresford,C.R., Goldberg,L. Smith, J.P. (1957)
Br J Pharmac Chemother 12, 107

Beuttner,G.R., Doherty,T.P., Patterson,L.K. (1983)
FEBS Lett 158, 143

Biedermann,G. & Schindler,P. (1957)
Acta Chem Scand 11, 731

Bielski,B.H.J. (1985) Phil Trans R Soc Lond B 311, 473

Bielski,B.H.J. (1978) Photochem Photobiol 28, 645

Bielski,B.H.J. (1983) in: "Oxygen Radicals and their Scavenger Systems" 1, p.1-17 (ed. G.Cohen & R.A.Greenwald) Elsevier

Bielski,B.H.J. & Gebicki,J.M. (1977) in: " Free Radicals in Biology" III, p.18-49 (ed. W.A. Pryor) Academic Press

Biemond,P., van Eijk,H.G., Swaak,A.J.G., Koster,J.F. (1984) J Clin Invest 73, 1576

Blake, D.R., Hall,N.D., Treby,D.A., Halliwell,B., Gutteridge,J.M.C. (1981a) Clin Sci 61, 483

Blake,D.R., Hall,N.D., Bacon,P.A., Dieppe,P.A., Halliwell,B., Gutteridge,J.M.C. (1981b) Lancet ii, 1141

Blake,D.R., Lunec,J., Athern,M., Ring,E.F.,
Bradfield,J.,Gutteridge,J.M.C. (1985)
Ann Rheum Dis 44, 183

Blake,D.R., Winyard,P., Lunec,J., Williams,A., Good,P.A., Crewes,S.J., Gutteridge,J.M.C., Rowely,D., Halliwell,B., Cornish,A., Hider,R.C. (1985) Q J Med 56, 345

Bolam,B.J. & Ulvik,R.J. (1987) Biochem J 243, 55

Borggaard,O.K.,Farver,O.,Anderson,V.S. (1971)
Acta Chem Scand 45, 3541

Borovanski,J. & Riley,P.A. (1983)
Eur J Cancer Clin Oncol 19, 91

Borovanski,J. & Riley,P.A. (1988)
Chem-Biol Interact in press

Borovanski,J. & Riley,P.A., Vrankova,E., Necas,E. (1985)
Neoplasma 32, 4

Bothwell,T.H. & Finch,C.A. (1962) in "Iron Metabolism"
p.440 Little, Brown & Co.

Boveris,A. & Chance,B. (1973) Biochem J 134, 707

Bradly,M.O. & Erickson,L.C. (1981) Biochim Biophys Acta 654, 135

Bränden,C.I., Jörnvall,H., Elkind,H., Furugren,B. (1975) in: " The Enzymes" 3rd ed. 11, p.103-190 (ed. P.Boyer) Academic Press N.Y.

Bremner,I., Cox,J.S.G., Moss,G.F. (1969) Carbohyd Res 11, 77

Bridges,K.R. & Hoffman,K.E. (1986) J Biol Chem 261, 14273

Brise,H. & Hallerberg,L. (1962) Acta Med Scand 171, 1

Bull,C., McClune,G.J., Fee,A.J. (1983) J Am Chem Soc 105, 5290

Bumbry,P.E. & Massey,V. (1976) in: "Methods in Enzymology" 10, p.463-474 (eds. R.W.Estabrook & M.E.Pullman) Academic Press N.Y.

Bulkley,B.J., Tanswell,A.K., Freemann,B.A. (1987) J Appl Physiol 63, 359

Bunting,H. (1949) Stain Tech 24, 109

Butler,P.G. ( ) PhD Thesis, University of Cambridge

Canagaratna,M.C.P. & Riley,P.A. (1975) J Cell Physiol 85, 271

Cantoni,O., Murray,D., Meyn,r.E. (1987) Chem-biol Interact 63, 29

Carter,P. (1971) Anal Biochem 40, 450

Carvill,I., Worwood,M., Jacobs,A. (1975) Nature 256, 328

Castor,L.N. (1968) J Cell Physiol 72, 161

Castranova,V., Wright,J.R., Colby,H.D., Miles,P.R. (1983) J Appl Physiol 54, 208

Chance,B. (1949) J Biol Chem 179, 1314

Chance,B. (1952) Arch Biochem Biophys 41, 416

Chance,B., Greenstein,D.S., Roughton,J.W. (1952) Arch Biochem Biophys 37, 301

Chance,B., Sies,H., Boveris,A. (1979) Physiol Rev 59, 527

Chapman,J.D., Reuvers,A.P., Borsa,J., Greenstock,C.L. (1973)'radiat Res 56, 291

Chaudierre,J. Wilhelmsen,E.C., Tappel,A.C.L. (1984) J Biol Chem 259, 1043

Cher,M. & Davidson,N. (1955) J Am Chem Soc 77, 793

Cook,J.D. (1970) J Lab Clin Med 76, 497

Cook,J.D. & Rummel,W. (1973) Am J Clin Nutr 29, 614

Coggle,J.E. (1983) in: "Biological Effects of Radiation", 2nd ed. Taylor & Francis Ltd. London

Cox,J.S.G., Kennedy,G.R., King,J., Marshall,P.R., Rutherford,D. (1971) J Pharm Pharmac 24, 513

Cox,J.S.G., King,R.E., Reynolds,G.F., (1965) Nature 207, 1202

Cox,J.S.G., Moss,G.F., Bremner,I., Reason,J. (1968) J Clin Path 21, 611

Crichton,R.R. (1984) Trends Biochem Sci 9, 283

Crichton,R.R., Charloteaux-Waters,M. (1987) Eur J Biochem 164, 485

Culling,C.F.A. (1974) in: "Handbook of Histopathological & Histochemical Techniques" 3rd ed p.267-268 Butterworth & Co. Ltd

Damluji,R. & Riley,P.A. (1979) Exp Cell Biol 47, 226

Das,S., Deeble,D.J. (1985) Naturforche 40, C 292

Dautry-Varsat,F., Ciechanover,A, Lodish,H.F. (1983) Proc Natl Acad Sci 80, 2258

Davies,M.J., Donkar,R., Dunster,C.A., Gee,C.A., Jonas,S.K., Willson,R.L. (1987) Biochem J 246, 725

Davies,M.J., Garlick,P.B., Shuter,S.L., Slater,T.F., Hearse,D.J. (1987a) Circulation 76, IV-196

Delgado,C.J. & Slobodian,E. (1972) Biochim Biophys Acta 268, 121

Drysdale,J.W., Arosio,P., Adelman,T., Hazard,J.T., Brooks,D. (1975) in: "Proteins of Iron Storage and Transport in Biochemistry and Medicine" p.356-366 (ed. R.R. Crichton) Am Elsevier Publ Co.

Dunford,H.B. (1982) Adv Inorg Biochem 4, 41

Durrant,P.J. & Durrant,B. (1970) in: "Introduction to Advanced Inorganic Chemistry" p.1064 Longman, London

Elkind,M.M. & Redpath,V.L. (1977) in: "Cancer, A Comprehensive Treatise" 6, p.51 (ed. F.F. Becker) Plenum Press

Elkind,M.M., Utsumi,H., Ben-Hur,E. (1987) Br J Cancer 55, 24

Fee,J.A. (1981) in: "Oxy-Radicals in Chemistry and Biology" p.205-231 (eds. J.Rodgers & E.L.Powers) Academic Press

Fee,J.A. (1982) Trends Biochem Sci 7, 84

Fee,J.A.,McClune,G.J., O'Neill,P., Fielden,E.M., (1981) Biochem Biophys Res Commun 100, 377

Fenton,H.J.H. & Jackson,A. (1899) J Chem Soc Transact 75, 1

Fielding,J. (1961) Br Med J 2, 279

Finch,C.A. & Heubers,H. (1982) N Engl J Med 306, 1520

Fisher,V. & Mason,R.P. (1986) Chem-Biol. Interact 57, 129

Flitter,W., Rowley,D.A., Halliwell,B. (1983) FEBS Lett 152, 310

Flöhe,L. (1971) Klin Wochenschr 49, 669

Flöhe,L., Loschen,G., Günzler,W.A., Eichele,E. (1972) Z Physiol Chem 353, 987

Floersheim,G.L. & Floersheim,P. (1986) Br J Radiol 59, 597 (1986)

Floyd,R.A. (1983) Arch Biochem Biophys 225, 263

Floyd,R.A. & Lewis,C.A. (1983) Biochemistry 22, 2649

Floyd,R.A., Soong,L.M., Waller,R.N., Stuart,M. (1967) Cancer Res 36, 2761

Forth,W. & Rummel,W. (1973) Physiol Rev. 53, 724

Freed,N. (1982) J Am Osteopath Assoc. 82, 115

Fricke,H. & Hart,E.J. (1966) in: "Radiation Dosimetry, Instumentation" 2, (eds. F.H.Attix & W.C.Roesch) Academic Press

Fridovich,I. (1983) Ann Rev Pharmacol Toxicol 23, 239

Fujita,S. & Steenken,S. (1981) J Am Chem Soc 105, 4380

Funk,F., Lenders,J.P., Crichton,R.R., Schneider,W. (1985) Eur J Biochem 152, 167

Gallagher,K.J. & Phillips,D.N. (1969) Chimica 23,465

Ganeshaguru,A.V., Hoffbrand,A.V., Gardy,R.O., Cerani,A. (1980) Biochem Pharmac 29, 1275

Garby,L., Irnell,L., Werner,I. (1969) Acta Med Scand 185, 113

Garlick,P.B., Davies,M.J., Hearse,D.J., Slater,T.F. (1987) Circ Res 61, 757

Gee,C.A., (1986) PhD Thesis Brunel

Gee,C.A., Kitteridge,K.J., Willson,R.L. (1984) Br J Radiol 57, 953

Geffner,J.R., Giorando,M., Palermo,M.S., Prat,A., Serebrinski,G.P., Isturiz,M.A. (1987) Clin Exp Immunol 69, 668

Gibbs,C.R. (1976) Anal Chem 48, 1197

Gilbert,B.C., King,D.M., Thomas,C.B. (1984) Carbohydr Res 125, 217

Goldberg,L. (1958) in: " Iron in Clinical Medicine" p.74 (eds. R.O.Wallerstein & S.R.Mettier) Univ of California Press: Berkeley

Goldstein,S., Czapski,G.,Cohen,H.,Meyerstein,D. (1988) JACS 110, 3903

Goscin,S.A. & Fridovich,I. (1973) Radiat Res 56, 565

Goto,K., Tamura,H., Nagayama,M. (1970) Inorg Chem 9, 963

Graf,E., Mahony,J.R., Bryant,R.G., Eaton,J.W. (1984) J Biol Chem 259, 3620

Grant,C.T. (1965) PhD Thesis Yale University

Greenwald,R.A. & Moy,W.W. (1980) Arthritis Rheum 23, 455

Gutteridge,J.M.C., Richmond,R., Halliwell,B. (1979)
Biochem J 184, 469

Haber,F. & Weiss,J. (1934)
J Proc Roy Soc (Lond) A, 147, 332

Halliwell,B. (1975) FEBS Lett 56, 34

Halliwell,B. (1975) FEBS Lett 86, 139

Halliwell,B. (1978) FEBS Lett 92, 321

Halliwell,B. (1982) Cell Biol Int Rep 6, 529

Halliwell,B. (1983) in: "Copper Proteins" 2,
CRC Press, Boca Raton,FL (ed. R.Lontie)

Halliwell,B., Gutteridge,J.M.C., Blake,D. (1985)
Phil Trans R Soc Lond B 311, 659

Halliwell,B. & Gutteridge,J.M.C. (1984) Biochem J 219, 1

Halliwell,B. & Gutteridge,J.M.C. (1985) in: "Free Radicals in Biology and Medicine" Oxford University Press

Hamstra,M.D., Block,M.H., Schockert.A.L. (1980)
JAMA 243, 1726

Harris,I. (1964) Nature 203, 30

Harrison,P.M. (1964) Ferritin and Haemosiderin in: "Iron Metabolism" (ed. F.Gross) Springer

Harrison,P.M. & Hoy,T.G. (1973) in: " Inorganic Chemistry" 1, p.253-277 (ed. G.I. Eichorn) Elsevier

Hawkins,C. & Perrin,D. (1962) J Chem Soc 1351

Hazra,D.K. & Steenken,S. (1983) J Am Chem Soc 105, 4380

Heubner,W. (1948) Nauyn-Schmiederbergs Arch Exp Pathol Pharamkol 205, 310

Hiller,K-O.& Asmus,K-D. (1981)
Int J Radiat Biol 40, 583

Hiller,K-O., Hodd,P.L., Willson,R.L. (1983)
Chem-Biol Interact 47, 293

Hirashi,H., Terano,A., Ota,S.I., Ivey,K.J., Sugimoto,T. (1987) Am J Physiol 253, G40

Hoard,J.L., Smith,G.S., Lind,M. (1961) in: "The Chemistry of the Coordination Compounds" p.296 (eds. S.Kirschner) Macmillan Press Ltd.

Hoe,S., Rowley,D.A., Halliwell,B. (1982)
Chem-Biol Interact 41, 75

Hoffmann,M.E. & Meneghini,R. (1979)
Photochem Photobiol 30, 151

Hoffmann,M.E., Mello-Fihlo,A.C., Meneghini,R. (1984)
Biochim Biophys Acta 781, 234

Holmes,R.S. & Masters C.J. (1972)
Arch Biochem Biophys 148 217

Horrocks,D.L. (1970) in: "The Current Status of Scintillation Counting" p.25-40 (ed. E.D.Bransome,Jr,M.D.) Grune & Stratton

Houk,J.C., Weil,R.L., Sharma,V.K., (1972)
Nature New Biol 240, 210

Hoy,T.J. & Harrison,P.M. (1976) Br J Haematol 33, 497

Iacopetta,B.A., Morgan,E.H., Yeoh,G.C.T. (1982)
Biochim Biophys Acta 687, 204

Ilan,Y. & Czapski,G. (1977) Biochim Biophys Acta 498, 386

Imaly,J.A., Chin,S.M., Linn,S. (1988) Science 240, 640

Irving,H. & Mellor,D. (1962) J Chem Soc 5222, 5232

Jacobs A. (1977) in: "Iron Metabolism" 51, p.91-106
Ciba Foundation Symposium Elsevier Excerpta Medica

Jacobs,G.B., Samuni,A., Czapski,G. (1985)
Int J Radiat Biol 47, 621

Janata,E. & Shuler,R.H. (1982) J Phys Chem 86, 2078

Jin,Yi. & Crichton,R.R. (1987) FEBS Lett 215, 41

Johansen,I. & Howard-Flanders,P. (1965)
Radiat Res 24, 184

Jörnvall,H. (1963) Proc Natl Acad Sci 70, 2295

Jörnvall,H. (1977) Eur J Biochem 72, 425

Jones,D.P., Eklöw,L. Thor,H., Orrenius,S. (1981) Arch Biochem Biophys 210, 505

Jones,D.P., Kennedy,F.G. (1983) in: "Fuctions of Glutathione: Biochemical, Physiological, Toxicoloogical and Clinical Aspects" p.109-116 (ed. A.Larrson et al) Raven Press

Jones,S.S. & Long,F.A.(1952a) J Phys Chem 56, 25

Kanakakorn,K., Cavill,I., Jacobs,A. (1973) Br J Haematol 25, 637

Kashiwagi,K., Tobe,T., Higashi,T. (1971) J Biochem 70, 785

Kellog,E.W. & Fridovich,I. (1977) J Biol Chem 252, 6721

Kenner,J. & Richards,g.N. (1956) J Chem Soc 2916

Klausner,R.D., Ashwell,G. van Renwonde,J., Harford,J.B., Bridge,K.R. (1983) Proc natl Acad Sci 80, 2263

Klinman,J.P. & Welsh,K. (1976) Biochem Biophys Res Commun 70, 878

Klug,A. & Rhodes,D. (1987) trends Biochem Sci 12, 465

Knight,R.J. & Sylvia,R.N. (1974) J Inorg Nucl Chem 36, 591

Kobayashi,N., Funayama,K., Koshiyama,M., Osa,T., Schirai,H., Hanabusa,K. (1983) J Chem Soc Chem Commun 915

Komov,V.P. & Schmelev, (1976) Biofizika 21, 799

Kontoghiorghes,G.J. (1987) Biochim Biophys Acta 924, 13

Koster,J.F. & Slee,R.G. (1986) FEBS Lett 199, 85

Kostrimina,N.A. (1974) Zhur Neorg Khim 19, 1322

Kroll,E., Pinching,G., Butler,F. (1952) Abs.122nd, Amer Chem Soc Meeting, Atlantic City, p.14

Kurimura,Y. & Kurimura,H. (1969) Bull Chem Soc Jap 42, 2238

Kurimura,Y. Ochiai,R., Matsuura,N. (1968)
Bull Chem Soc Jap 41, 2234

Lambeth,D.O., Erickson,G.R., Yorek,M.A., Ray,p.D. (1982)
Biochim Biophys Acta 719, 501

Land,E.J. & Prutz, W.A. (1979) Int J radiat Biol 36, 75

Lanform,H. & Nielsen,S.O. (1957) J Am Chem Soc 79

Langer,E.E., Haining,R.G., Labbe,r.F., Jacobs,A.,
Crosby,E.F., Finch,C.A. (1972) Blood 40, 112

Langford,C.H., Wong,S.M., Underdown,A.W.
Can J Chem 59, 181

Larramendy,M., Mello Fihlo,A.C., Leme Martins, E.A.,
Meneghini,R. (1987) Mutation Research 178, 57

Latimer,W.M. (1952) "Oxidative Potentials" Prentice-Hall,
Englewood Cliffs, N.J.

Lavelli,F., McAdam,M.E., Fielden,e.M., Puget,K.,
Michelson,A.M. (1977) Biochem J 3

Lazarow,P.B. & deDuve (1973) J Cell Biol 59, 491 & 507

Ledermann,H.M., Cohen, A., Lee,J.W.W., Freedmen,M.H.,
Gelfand,E.W. (1984) Blood 64, 748

Lee,G.R., Nacht,S., Christensen,D., Hansen,S.P.,
Cartwright,G.E. (1969) Proc Soc Exp Biol Med 131, 918

Lesko,S.A. (1982) Biochemistry 21, 5010

Lewin,S. (1976) in: "Vitamin C: Its Molecular Biology and
medical Potential" p.75-101 Orlando,F.L. Academic Press

Link,E.M. & Riley,P.A. (1988) Biochem J 249, 391

Lison,L. (1936) C.R. Soc Biol 191, 900

Lloyd,K.N. & Williams,P. (1970) Br Med J 2,323

Loewenstein,W.R. (1968) Dev Biol Suppl 2, 151

Lorentzen,R.J. & Ts'O,P.P. (1977) Biochemistry 16, 1467

Loven,J.M. (1884) J F Prakt Chem 29, 366

Massie,H.R., Samis,H.V., Baird,M.B. (1972)
Biochim Biophys Acta 272, 539

Marshall,P.R. & Rutherford,D. (1971)
J Colloid & Interface Sci 37, 390

Martin,W.J. (1984) Am Rev Respir Dis 130, 209

Matsuyama,A. & Nagata,C. (1972) in: "Topics in Chemical Carcinogenesis" p.35 (eds. W. Nakahara, S. Takayama, T. Sugimura, S.Odashima) Univ Park Press, Baltimore

Matthews,A.P. & Walker,S. (1909) J Biol Chem 6, 299

McCardy,P.R. (1965) J Am Med Ass 191, 859

McClune,G.J., Fee,J.A., McClusky,G.A., Groves,J.T. (1977)
J am Chem soc 99, 5220

McCord,J.M. & Day,E.D. (1978) FEBS Lett 86, 139

Meingassner,J.G. & Heyworth,P.G. (1982)
J Parasitology 68, 1163

Mello Fihlo,A.C. & Meneghini,R. (1984)
Biochim Biophys Acta 781, 56

Menighini,R. & Hoffmann,M.E. (1980)
Biocim Biophys Acta 608, 167

Misra,H.P. (1974) J Biol Chem 249, 2151

Mittal,M.M., Bhargava,S.P., Scharma,M.L. (1969)
J Ass Physicians India 17, 45

Modell,B., Letsky,E.A., Flynn,D.M., Peto,R., Weatherhall,D.J. (1982) Br Med J 284, 1081

Mondon,K.J. (1985) PhD Thesis Brunel

Moshlin,S. & Schnider,U. (1963) N Engl J Med 269, 57

Moster,L.J., deJong,G., Koster,J.F., van Euk,H.G. (1986)
Int J Biochem 18, 1061

Munro,H.N. & Linder,M.C. (1978) Physiol Rev 58, 317

Nathan,C.F., Arrick,B.A., Murray,H.W., De Santis,N.M., Cohn,Z.A. (1981) J Exp Med 153, 766

Naukova,E. (1965) in: "Complexes as agents for the treatment of lime chlorosis in plants" Dumka, Kiev

Newcombe,R. (1967) Postgrad Med J 43, 372

Nicholls,P. (1962) Biochim Biophys Acta 60, 217

Nicholls,D. (1973) Iron, in: "Comprehensive Inorganic Chemistry" 3, p.979-1051 (eds. J.C.Bailar, H.J.Emelens, R.Nyholm, A.G.Trotman-Dickenson) Pergamon Press

Nicholls,P. & Schonbaum,G.R. (1963) Catalases, in: "The Enzymes" 8, p.147-222 2nd ed,. (ed. Boyer, Lardy, Mybräck) Academic Press

Nielands,J.B. (1972) Struct Bonding 2, 145

Nienhuis,A.W. (1981) N Engl J Med 304, 170

Nissim,J.A. (1947) Lancet ii, 49

Nyholm,R.S. & Tobe,M.L. (1963) Adv Inorg Chem Radiochem 5, 1

O'Connell,M.J., Ward,R.J., Baum,H., Peters,T.J. (1985) Biochem J 229, 135 .

Olivieri,N.F., Buneic,J.R., Chew,E., Gallant,T., Harrison,R.V., Keenan,N., Logan,W., Mitchell,D., Ricci,R.V., Skarf,B., Taylor,M., Freedman,M.H. (1986) N Engl J Med 314, 869

Olson,M.J. (1988) J Toxicol Environ Health 23, 407

Packer,J.E., Mahood,J.S., Willson,R.L., Wolfenden,B.S. (1981) J Radiat Biol 39, 135

Paques,E.P., Paques,A., Crichton,R.R. (1980) Eur J Biochem 107, 447

Parshad,R., Scandford,K.K., Jones,G.M., Torone,R.E. (1978) Proc natl Acad Sci 75, 1830

Pasternak,R.F., & Halliwell, B. (1979) J Am Chem Soc 101, 1026

Pasternak,R.F., Skowronek,W.R.,Jr (1979) J Inorg Biochem 11, 261

Pastore,E. & Friedken,M. (1962) J Biol Chem 237, 3802

Pauling,L. (1960) in: "Nature of The Chemical Bond" 3rd ed., Ithaca, Cornell Univ Press

Pearse,A.G.E. (1972) in: "Histochemistry, Theoretical & Applied" 2, 3rd ed. App 28 J & A Churchill Livingstone

Peretz,P., Solomon,D., Weinraub,D., Faraggi,M. (1982) Int J Radiat Biol 42, 449

Perls,M. (1867) Virchows Arch 39, 42

Perrin,D. (1959) Rev Appl Chem 9 257

Pierre, T.G.St., Dickson,D.P.E., Kirkwood,J.K., Ward,R.S., Peters,T.J. (1987) Biochim Biophys Acta 924, 447

Pitt,D. (1975) in: "Lysosomes & Cell Function" p.1-24 Integrated Themes in Biology, Longman

Pratten, M.K. & Lloyd,J.B. (1979) Biochem J 180, 567

Prutz,W.A., Butler,J., Land,E.J., Swallow,A.J. (1980) Biochem Biophys Res Commun 96, 408

Prutz,W.A, Land,E.J., Sloper,R.W. (1981) J Chem Soc Farad Trans I 77, 281

Prutz, W.A, Siebert,F., Butler,J., Land,E.J., Menez,A., Montenay-Gerastier,T. (1982) Biochim Biopys Acta 704, 139

Racker,E. & Krimski,I. (1947) J Exp Med 85, 715

Reddy, M.D. & Lewis,M. (1969) Arthritis & Rheumatism 12, 454

Rees,T.D & Orth,C.F. (1986) J Periodontol 57, 689

Rhaesse,H.J. & Freese,E. (1968) Biochim Biophys Acta 155, 476

Riley,P.A., Canagaratna,C., Bowler,L.M., Latter,T., Bowler,E. (1973) Lab Practice 22, 116

Roots,R. & Okada,S. (1975) Radiat Res 64, 306

Rowely,D.A. & Halliwell,B. (1982) FEBS Lett 138, 33

Rowley,B. & Sweeney,G.D. (1984) Can J Biochem Cell Biol 62, 1293

Rubbo,S., Albert,A., Gibson,M. (1950) Br J Exp Path 31, 425

Rubin,R. & Farber,J.L. (1984) Arch Biochem Biophys 228, 450

Rubin,R. & Farber,J.L. (1984)
Arch Biochem Biophys 228, 450

Saha,N. & Sigel,H. (1982) J Am Chem Soc 104, 4100

Samuni,A., Bump,E.A., Mitchell,J.B., Brown,J.M. (1986)
Int J Radiat Biol 49, 77

Shilt,A.A. & Hoyle,W.C. (1967) Anal Chem 39, 114

Schirazi,M.F., Aronson,N.N., Dean,R.T. (1982)
J Cell Sci 57, 115

Schonbaum,G.R. & Chance,B. (1976) Catalase, in: "The Enzymes" 2nd ed XIII p.363-408 (ed P.D.Boyer)
Academic Press

Schuchmann,M.N. & v.Sonntag,C. (1978a)
J Chem Soc Perkin Trans II, 1958

Schuchmann,M.N. & v.Sonntag,C. (1978b)
Z. Naturforsch 336, 329

Schugar,H.J., Hubbard,A.T., Anson,F.C., Gray,H.B. (1969)
J Am Chem Soc 91, 71

Schwarzenbach,G. & Heller,J. (1951)
Helv Chim Acta 34, 576

Schweiz,H. (1969) Biopolymers 18, 101

Scott,J. (1962) Br Med J 2, 480

Searle,A.J.F. & Tomasi,T. (1982)
J Inorg Biochem 17, 161

Selden, L., Owen,M., Hopkins,J.M.P., Peters,T.J. (1980)
Br J Haematol 44, 593

Silgar,S.G., Kennedy,K.A., Pearson, D.C. (1982)
in: "Oxidases & Related Redox Systems" p.837-856
(eds. T.E.King, H.S.Mason, M.Morrison)
Pergamon Press

Sillen,L. & Martell,A. (1964) In: "Stability Constants of metal ion Complexes" The Chemical Society

Simon,R.H., Scoggin,C.H., Patterson,D. (1981)
J Biol Chem 256, 7181

Simon,P. Aug,K.S., Meyrier,A., Allain,P., Mauras,Y.
(1983) Lancet ii, 512

Simpson,R.J. & Peters,T.J. (1987)
Biochim Biophys Acta 898, 187

Sinaceur,J. Abumurad,C., Nordmann,J., Nordmann,R. (1983)
Biochem Pharmacol 32, 2371

Sinaceur,J. Riberi,C., Nordmann,J., Nordmann,R. (1984)
Biochem Pharmacol 33, 1693

Slater,T.F. & Riley,P.A. (1966) Nature 209, 151

Smith,H., Chapman,I., Marlow,C. (1969) Nature 222, 676

Spitz,D.R., Dewey,W.C., Li,G.C. (1987)
J Cell Physiol 131, 364

Stier,A., Reitz,I., Sackmann,E. (1972)
Naunyn-Schmeideberg's Arch Pharmacol 274, 189

Starke,P.E. & Farber,J.L. (1985) J Biol Chem 260, 86

Stoker,M.P.G. & Rubin,H. (1967) Nature 215, 171

Stookey,L.L. (1970) Anal Chem 42, 779

Sullivan, T.J. (1985)
in: "Imferon in Clinical Medicine" 3rd ed publ Fisons PLc

Sund,H. & Theorell,H. (1963) in: "The Enzymes" VII p.25
(ed. P.D. Boyer) Academic Press

Tamura,M. & Yamzaki,I. (1972) J Biochem 71, 311

Thacker,J., Strtch,A., Goodhead,D.T. (1982)
Radiat Res 92, 343

Theofilopoulus,A.N. & Dixon,F.J. (1982)
Am J Pathol 108, 321

Thomas,G.E., Marchouse,L.A., Aust, S.D. (1985)
J Biol Chem 260, 3275

Tien,M., Morehouse,L.A., Bucher,J.R., Aust,S.D. (1983)
Arch Biochem Biophys 218, 450

Todaro,G.J., Lazer,G.K., Green,H. (1965)
J Cell Comp Physiol 66, 325

Treffy,A. & Harrison,P.M. (1979) Biochem J 181, 709

Trowell,O.A. (1966) in: "Cells & Tissues in Culture" 3 p.76-80 (ed. E.N. Willmer) Academic Press

Tullis,D.T. (1987) Trends Biochem Sci 12, 297

Undienfriend,S., Clark,C.T., Axelrod,J., Brodie,B.B. (1954) J Biol chem 208, 731

Varma,S.D., Ets,T.K., richards,R.D. (1977) Opthalmic Res 9,421

Veillon,C. & Sytkowsi,A.J. (1975) Biochem Biophys Res Commun 67, 1494

von Sonntag,C. (1987) in: "The chemical basis of radiation biology" p.117 Taylor Francis

Walling,C. (1982) in: "Oxidases & Related Redox Systems" p.85-97 (eds. T.E.King, H.S.Mason, M.Morrison) Pergamon Press

Wang,R.J., Ananthaswamy,H.N., Nixon,B.T., Hartman,P.S., Eisenstark,A. (1980) Radiat Res 82, 269

Ward,J.F. (1975) Adv Radiat Biol 5, 182

Ward,J.F., Blakely,W.F., Moberly,J.B. (1983) Radiat Res 94, 629

Ward,J.F., Blakely,W.F., Joner,E.I. (1985) Radiat Res 103, 383

Ward,J.F., Evans,J,W., Limoli,C.L., Calabro-Jones,P.M. (1987) Br J Cancer 55, 105

Welsh,M.J., Shasby,D.M., Husted,R.M. (1985) J Clin Invest 76, 1155

Wenham,R.B. (1984) Briefing Report for the Imferon Workshop; Fisons Plc

Weinstein,J. & Bielski, B.H.J. (1979) J Am Chem Soc

White,G.P., Bailey-Wood,R., Jacobs,A. (1976) Clin Sci Mol med 50, 145

WHO Technical Report Series no. 182 (1959) "Iron deficiency Anaemia" Report of a study group Geneva

Wigglesworth,J.M. & Baum,H. (1980) in: " Iron in Biochemistry & Medicine II" p.29-86 (ed. A.Jacobs & M.Worwood) Academic Press

Wills,E.D. (1969) Biochem J 113, 315

Wills,E.D. & Wilkinson,A.E. (1967)
Int J Radiat Biol 13, 45

Willson,R.L. (1970) Int J Radiat Biol 17, 349

Willson,R.L. (1977) in: "Iron Metabolism" p.331-354
Ciba Foundation Symposium Elsevier,Excerpta Medica

Willson,R.L. (1982) in: "Free Radical Lipid Peroxidation & Cancer" p.275-303 NFCR Cancer Symposium 1
Academic Press

Willson,R.L. (1985) in: "Oxidative Stress" p.41-72
(ed. H.Sies) Academic Press

Willson,R.L. (Zn Review, 1988 in press?)

Willson,R.L., Dunster,C.A., Forni,L.G., Gee,C.A., Kitteridge,K.J. (1985)
Phil Trans R Soc Lond BO, 1

Willson, R.L. & Searle, A.J.F. (1975) Nature, 225, 498

Winterbourn,C.C. (1981) Biochem J 198, 125

Ziegler-Sylakakis,K., Andrae,U. (1987) Mutat Res 192, 65

Zigler,J.S.,Jr, Jernigan,H.M.,Jr, Garland,D., Reddy,V.N.
(1985) Arch Biochem Biophys 215, 163

Zimm,B.H. & LeBret,M. (1983)
J Biomol Struct Dynamics 1, 461